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## Protection of Environment

## 40

PART 790 TO END

**Revised as of July 1, 1989**

National Archives and Records Administration
1985

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## Protection of Environment

## 40

PART 790 TO END

**Revised as of July 1, 1989**

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Title 40, Parts 700 to 789
and
Title 40, Part 790 to End

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*Cite this Code:* **CFR**

*To cite the regulations in this volume use title, part and section number. Thus,* 40 CFR 790.1 *refers to Title 40, Part 790, section 1.*

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# Explanation

The Code of Federal Regulations is a codification of the general and permanent rules published in the Federal Register by the Executive departments and agencies of the Federal Government. The Code is divided into 50 titles which represent broad areas subject to Federal regulation. Each title is divided into chapters which usually bear the name of the issuing agency. Each chapter is further subdivided into parts covering specific regulatory areas.

## ISSUE DATES

Each volume of the Code is revised at least once each calendar year and issued on a quarterly basis approximately as follows:

Title 1 through Title 16............................................................as of January 1
Title 17 through Title 27............................................................as of April 1
Title 28 through Title 41............................................................as of July 1
Title 42 through Title 50............................................................as of October 1

The appropriate revision date is printed on the cover of each volume.

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The contents of the Federal Register are required to be judicially noticed (44 U.S.C. 1507). The Code of Federal Regulations is prima facie evidence of the text of the original documents (44 U.S.C. 1510).

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Each volume of the Code contains amendments published in the Federal Register since the last revision of that volume of the Code. Source citations for the regulations are referred to by volume number and page number of the Federal Register and date of publication. Publication dates and effective dates are usually not the same and care must be exercised by the user in determining the actual effective date. In instances where the effective date is beyond the cut-off date for the Code a note has been inserted to reflect the future effective date. In those instances where a regulation published in the Federal Register states a date certain for expiration, an appropriate note will be inserted following the text.

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The Paperwork Reduction Act of 1980 (Pub. L. 96-511) requires Federal agencies to display an OMB control number with their information collection request. Many agencies have begun publishing numerous OMB control numbers as amendments to existing regulations in the CFR. These OMB numbers are placed as close as possible to the applicable recordkeeping or reporting requirements.

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(c) The incorporating document is drafted and submitted for publication in accordance with 1 CFR Part 51.

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Martha L. Girard,
*Acting Director,*
*Office of the Federal Register.*

*July 1, 1989.*

# THIS TITLE

Title 40—PROTECTION OF ENVIRONMENT is composed of fourteen volumes. The parts in these volumes are arranged in the following order: Parts 1-51, Part 52, Parts 53-60, Parts 61-80, Parts 81-85, Parts 86-99, Parts 100-149, Parts 150-189, Parts 190-299, Parts 300-399, Parts 400-424, Parts 425-699, Parts 700-789 and Part 790 to End. A derivation table appears in the volume containing Parts 1-51. The contents of these volumes represent all current regulations codified under this title of the CFR as of July 1, 1989.

Chapter I—Environmental Protection Agency appears in all fourteen volumes. A Pesticide Tolerance Commodity/Chemical Index appears in Parts 150-189. A Toxic Substances Chemical—CAS Number Index appears in Parts 700-789 and Part 790 to End. Distribution Tables appear in the volumes containing Parts 100-149 and Parts 190-299. Redesignation Tables appear in the volumes containing Parts 1-51, Parts 81-85, Parts 86-99, Parts 150-189, Parts 700-789 and Part 790 to End. Regulations issued by the Council on Environmental Quality appear in the volume containing Part 790 to End.

For this volume, Tracey L. Gregg was Chief Editor. The Code of Federal Regulations publication program is under the direction of Richard L. Claypoole, assisted by Ruth C. Pontius.

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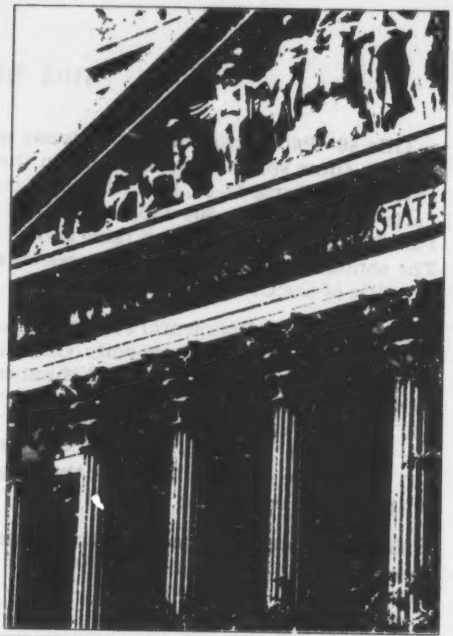

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# Title 40—Protection of Environment

(This book contains Part 790 to End)

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# SUBCHAPTER R—TOXIC SUBSTANCES CONTROL ACT—(Continued)

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AUTHORITY: 15 U.S.C. 2603.

## Subpart A—General Provisions

**§ 790.1 Scope, purpose, and authority.**

(a) This part establishes procedures for gathering information, conducting negotiations, and developing and implementing test rules or consent agreements on chemical substances and mixtures under section 4 of TSCA.

(b) Section 4 of the Act authorizes EPA to require manufacturers and processors of chemical substances and mixtures to test these chemicals to determine whether they have adverse health or environmental effects. Section 4 (a) empowers the Agency to promulgate rules which require such testing. In addition, EPA has implied authority to enter into enforceable consent agreements requiring testing where they provide procedural safeguards equivalent to those that apply where testing is conducted by rule.

(c) EPA intends to use enforceable consent agreements to accomplish testing where a consensus exists among EPA, affected manufacturers and/or processors, and interested members of the public concerning the need for and scope of testing. If such a consensus does not exist and the Agency believes that it can make the findings specified in section 4(a), EPA will initiate proceedings to promulgate test rules which will be codified in Part 799 of this chapter.

(d) Appendix A to this part presents timetables for various steps in the evaluation of chemicals under consideration for testing, the initiation and completion of negotiations to develop consent agreements, and the proposal and promulgation of test rules. All deadlines which are imposed by the Act are binding on EPA and will be observed by the Agency. The remaining deadlines represent target dates that EPA intends to meet.

[51 FR 23712, June 30, 1986]

### § 790.2 Applicability.

This part is applicable to manufacturers and processors of chemical substances or mixtures who are subject to the testing requirements of a consent agreement or a rule under section 4(a) of the Act. The procedures for test rules are applicable to each test rule in Part 799 or this chapter unless otherwise stated in specific test rules in Part 799 of this chapter.

[51 FR 23712, June 30, 1986]

### § 790.3 Definitions.

Terms defined in the Act and not explicitly defined herein are used with the meaning given in the Act. For the purpose of this part:

"Act" means the Toxic Substances Control Act, 15 U.S.C. 2601 *et seq.*

"Additive" means a chemical substance that is intentionally added to another chemical substance to improve its stability or impart some other desirable quality.

"Chemical" means a chemical substance or mixture.

"Consortium" means an association of manufacturers and/or processors who have made an agreement to jointly sponsor testing.

"EPA" means the U.S. Environmental Protection Agency.

"Equivalence data" means chemical data or biological test data intended to show that two substances or mixtures are equivalent.

"Equivalent" means that a chemical substance or mixture is able to represent or substitute for another in a test or series of tests, and that the data from one substance can be used to make scientific and regulatory decisions concerning the other substance.

"Exemption" means an exemption from a testing requirement of a test rule promulgated under section 4 of the Act and Part 799 of this chapter.

"Impurity" means a chemical substance which is unintentionally present with another chemical substance.

"Joint sponsor" means a person who sponsors testing pursuant to section 4(b)(3)(A) of the Act.

"Joint sponsorship" means the sponsorship of testing by two or more persons in accordance with section 4(b)(3)(A) of the Act.

"Person" means an individual, partnership, corporation, association, scientific or academic establishment, or organizational unit thereof, and any other legal entity.

"Principal sponsor" means an individual sponsor or the joint sponsor who assumes primary responsibility for the direction of a study and for oral and written communication with EPA.

"Protocol" means the plan and procedures which are to be followed in conducting a test.

"Reimbursement period" refers to a period that begins when the data from the last non-duplicative test to be completed under a test rule are submitted to EPA and ends after an amount of time equal to that which had been required to develop data or after five years, whichever is later.

"Sponsor" means the person or persons who design, direct and finance the testing of a substance or mixture.

"Test substance" means the form of chemical substance or mixture that is specified for use in testing.

[49 FR 39782, Oct. 10, 1984, as amended at 51 FR 23712, June 30, 1986]

### § 790.5 Submission of information.

(a) All submissions to EPA under this part must bear the Code of Federal Regulations (CFR) section number of the subject chemical test rule, e.g., § 799.4400 1,1,1-Trichloroethane, or indicate the identity of the consent agreement. For all submissions under this part, six copies must be provided to EPA.

(b) Submissions containing both confidential business information or nonconfidential business information must be addressed to: TSCA Document Processing Center (TS-790), Rm L-100, Office of Toxic Substances, Environmental Protection Agency, 401 M St., SW., Washington, DC 20460. Attention: TSCA Section 4.

(c) [Reserved]

(d) In addition, a copy of the cover memo for all submissions must be addressed to: Director, Office of Compliance Monitoring (EN-342), Office of Pesticides and Toxic Substances, Environmental Protection Agency, 401 M St., SW., Washington, DC 20460.

(Approved by the Office of Management and Budget under control number 2070-0033)

[50 FR 20656, May 17, 1985, as amended at 51 FR 23712, June 30, 1986; 53 FR 12525, Apr. 15, 1988]

### § 790.7 Confidentiality.

(a) Any person subject to the requirements of a consent agreement or a test rule under section 4 of the Act may assert a claim of confidentiality for certain information submitted to EPA in response to the consent agreement or the test rule. Any information claimed as confidential will be treated in accordance with the procedures in Part 2 of this title and section 14 of the Act. Failure to assert a claim of confidentiality at the time the information is submitted will result in the information being made available to the public without further notice to the submitter.

(b) A claim of confidentiality must be asserted by circling or otherwise marking the specific information claimed as confidential and designating it with the words "confidential business information," "trade secret," or another appropriate phrase indicating its confidential character.

(c) If a person asserts a claim of confidentiality for study plan information described in §§ 790.50(c)(1)(iii)(D), (iv), (v), and (vi) and 790.62(b)(6), (7), (8), (9), and (10), the person must provide a detailed written substantiation of the claim by answering the questions in this paragraph. Failure to provide written substantiation at the time the study plan information is submitted will be considered a waiver of the claim of confidentiality, and the study plan information will be disclosed to the public without further notice.

(1) Would disclosure of the study plan information disclose processes used in the manufacture or processing of a chemical substance or mixture? Describe how this would occur.

(2) Would disclosure of the study plan information disclose the portion of a mixture comprised by any of the substances in the mixture? Describe how this would occur.

(3) What harmful effects to your competitive position, if any, do you think would result from disclosure of this information? How would a competitor use such information? How substantial would the harmful effects be? What is the causal relationship between disclosure and the harmful effects?

(4) For what period of time should confidential treatment be given? Until a specific date, the occurrence of a specific event, or permanently? Why?

(5) What measures have you taken to guard against disclosure of this information to others?

(6) To what extent has this information been disclosed to others? What precautions have been taken in connection with such disclosures?

(7) Has this information been disclosed to the public in any forms? Describe the circumstances.

(8) Has the information been disclosed in a patent?

(9) Has EPA, another Federal agency, or any Federal court made any pertinent confidentiality determination regarding this information? If so, copies of such determinations must be included in the substantiation.

(d) If the substantiation provided under paragraph (c) of this section contains information which the submitter considers confidential, the submitter must assert a separate claim of confidentiality for that information at the time of submission in accordance with paragraph (b) of this section.

[49 FR 39782, Oct. 10, 1984, as amended at 51 FR 23713, June 30, 1986]

## Subpart B—Procedures for Developing Consent Agreements and Test Rules

SOURCE: 51 FR 23713, June 30, 1986, unless otherwise noted.

### § 790.20 Recommendation and designation of testing candidates by the ITC.

(a) *Recommendations with intent to designate.* The ITC has advised EPA that it will discharge its responsibilities under section 4(e) of the Act in the following manner:

(1) When the ITC identifies a chemical substance or mixture that it believes should receive expedited consideration by EPA for testing, the ITC may add the substance or mixture to its list of chemicals recommended for testing and include a statement that the ITC intends to designate the substance or mixture for action by EPA in accordance with section 4(e)(1)(B) of the Act.

(2) Chemical substances or mixtures selected for expedited review under paragraph (a)(1) of this section may, at a later time, be designated for EPA action within 12 months of such designation. The ITC's subsequent decision would be based on the ITC's review of TSCA sections 8(a) and 8(d) data and other relevant information.

(3) Where the ITC concludes that a substance or mixture warrants testing consideration but that expedited EPA review of testing needs is not justified, the ITC will add the substance or mixture to its list of testing recommendations without expressing an intent to designate the substance or mixture for EPA action in accordance with section 4(e)(1)(B) of the Act.

(4) The ITC reserves its right to designate any chemical that it determines the Agency should, within 12 months of the date first designated, initiate a proceeding under section 4(a) of the Act.

(b) *EPA consideration of ITC recommendations.* (1) Where a substance or mixture is designated for EPA action under section 4(e)(1)(B) of the Act, the Agency will take either one of the following actions within 12 months after receiving the ITC designation:

(i) Initiate rulemaking proceedings under section 4(a) of the Act.

(ii) Publish a FEDERAL REGISTER notice explaining the Agency's reasons for not initiating such rulemaking proceedings. EPA may conclude that rulemaking proceedings under section 4(a) of the Act are unnecessary if it determines that the findings specified in section 4(a) of the Act cannot be made or if the Agency has entered into a consent agreement requiring testing in accordance with the provisions of this Subpart.

(2) Where a substance or mixture has been recommended for testing by the ITC without an intent to designate, EPA will use its best efforts to act on the ITC's recommendations as rapidly as possible consistent with its other priorities and responsiblities. EPA may respond to the ITC's recommendations either by:

(i) Initiating rulemaking proceedings under section 4(a) of the Act.

(ii) Publishing a FEDERAL REGISTER notice explaining the Agency's reasons for concluding that testing is unnecessary.

(iii) Entering into a consent agreement in accordance with this subpart.

### § 790.22 Procedures for gathering information and negotiating consent agreements on chemicals which the ITC has recommended for testing with an intent to designate.

(a) *Preliminary EPA evaluation.* Following receipt of an ITC report containing a recommendation with an intent to designate, EPA will use the following procedure for completing a preliminary evaluation of testing needs. Appendix A[1] to this part presents the schedule that EPA intends to follow for this purpose.

(1) EPA will publish the ITC report in the FEDERAL REGISTER and announce that interested persons have 30 days to submit comments on the ITC's testing recommendations.

(2) EPA will publish a FEDERAL REGISTER notice adding all ITC-recommended chemicals to the automatic reporting provisions of its rules under sections 8(a) and 8(d) of the Act (40 CFR Parts 712 and 716).

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[1] Editorial Note: Appendix A appears at the end of Subpart E.

(3) EPA will hold a public "focus meeting" to discuss the ITC's testing recommendations and obtain comments and information from interested parties.

(4) EPA will evaluate submissions received under the sections 8(a) and 8(d) reporting requirements, comments filed on the ITC's recommendations, and other information and data compiled by the Agency.

(5) EPA will make a preliminary staff determination of the need for testing and, where testing appears warranted, will tentatively select the studies to be performed.

(6) EPA will hold a public meeting to announce its preliminary testing determinations.

(b) *Negotiation procedures for consent agreements.* Where EPA believes that testing is necessary, the Agency will explore whether a consent agreement can be negotiated that satisfies the testing needs identified by the Agency. EPA will use the following procedures for negotiating, formulating and accepting consent agreements. Appendix A[1] to this part presents the schedule that EPA intends to follow for this purpose.

(1) In the FEDERAL REGISTER notice described in paragraph (a)(1) of this section, EPA will explain its procedures and timetable for negotiating consent agreements and invite persons interested in participating in or monitoring negotiations to contact the Agency in writing.

(2) Persons who respond to EPA's notice by the announced date of the Agency's course-setting meeting will be deemed "interested parties" for purposes of any negotiations that EPA conducts.

(3) Following the course-setting meeting announcing EPA's preliminary testing determinations, the Agency will meet with manufacturers, processors and other interested parties for the purpose of attempting to negotiate a consent agreement. To facilitate attendance at these meetings, EPA will contact all interested parties who have expressed a desire to participate in or monitor negotiations under paragraph (b)(2) of this section and advise them of meeting dates.

(4) All negotiating meetings will be open to members of the public. The minutes of each meeting will be prepared by EPA. Meeting minutes, testing proposals, background documents and other materials exchanged at or prepared for negotiating meetings will be included in the public file established by EPA on each ITC-recommended chemical. Materials in this file will be made available for inspection in the OPTS Reading Room during EPA working hours.

(5) While negotiations are underway, EPA will promptly circulate meeting minutes, testing proposals, correspondence and other relevant materials to interested parties who expressed a desire to participate in or monitor negotiations pursuant to paragraph (b)(2) of this section.

(6) As negotiations progress, EPA will make a tentative decision either to proceed with formulation of a consent agreement or to initiate rulemaking. EPA will terminate negotiations after 10 weeks and proceed with rulemaking unless negotiations are likely to result in a draft consent agreement within 4 additional weeks. By the end of this 4-week period, EPA either will have prepared a draft consent agreement reflecting the apparent consensus of the parties or will terminate negotiations and proceed with rulemaking. If EPA decides to proceed with rulemaking, no further opportunity for negotiations will be provided. EPA will promptly send written notice to all interested parties of the termination of negotiations.

(7) Where EPA prepares a draft consent agreement, it will be circulated for comment to all interested parties who expressed a desire to participate in or monitor negotiations under paragraph (b)(2) of this section. A period of 4 weeks will be provided for submitting comments or written objections under § 790.24(a).

(8) If necessary, EPA will hold a public meeting to discuss comments on the draft consent agreement and to determine whether revisions in the agreement are appropriate.

(9) Where a consensus exists concerning the contents of a draft consent agreement, it will be circulated to EPA

management and interested parties for final approval and signature.

(10) Upon final approval of a consent agreement, EPA will publish a FEDERAL REGISTER notice that summarizes the agreement, describes the ITC recommendations for the test substance, outlines the chemical's use and exposure characteristics, and explains the background, objectives and rationale of the testing to be conducted, and codifies in Subpart C of Part 799 the name of the substance(s) to be tested and the citation to the FEDERAL REGISTER notice of the agreement.

### § 790.24 Criteria for determining whether a consensus exists concerning the provisions of a draft consent agreement.

(a) EPA will enter into consent agreements only where there is a consensus among the Agency, one or more manufacturers and/or processors who agree to conduct or sponsor the testing, and all other interested parties who identify themselves in accordance with § 790.22(b)(2). EPA will not enter into a consent agreement in either of the following circumstances:

(1) EPA and affected manufacturers and/or processors cannot reach a consensus on the testing requirements or other provisions to be included in the consent agreement.

(2) A draft consent agreement is considered inadequate by other interested parties who, pursuant to § 790.22(b)(2), have asked to participate in or monitor negotiations; and these parties have submitted timely written objections to the draft consent agreement which provide a specific explanation of the grounds on which the draft agreement is objectionable.

(b) EPA may reject objections described in paragraph (a)(2) of this section only where the Agency concludes the objections are either:

(1) Not made in good faith.

(2) Untimely.

(3) Do not involve the adequacy of the proposed testing program or other features of the agreement that may affect EPA's ability to fulfill the goals and purposes of the Act.

(4) Not accompanied by a specific explanation of the grounds on which the draft agreement is considered objectionable.

(c) The unwillingness of some manufacturers and/or processors of a prospective test chemical to sign the draft consent agreement does not, in itself, establish a lack of consensus if EPA concludes that those manufacturers and/or processors who are prepared to sign the agreement are capable of accomplishing the testing to be required and that the draft agreement will achieve the purposes of the Act in all other respects.

### § 790.26 Initiation and completion of rulemaking proceedings on ITC-designated chemicals.

(a) Where EPA concludes that a consensus does not exist concerning the provisions of a draft consent agreement and that the findings specified by section 4(a) can be made, the Agency will proceed with rulemaking under section 4(a) of TSCA.

(b) When EPA decides to proceed with rulemaking under paragraph (a) of this section, the Agency intends to publish a rulemaking proposal and a final rule or a notice terminating the rulemaking proceeding in accordance with the schedule specified in Appendix A[1] to this part.

(c) Where the testing recommendations of the ITC raise unusually complex and novel issues that require additional Agency review and opportunity for public comment, the Agency may publish an Advance Notice of Proposed Rulemaking (ANPR). The schedule that EPA intends to follow for rulemaking proceedings initiated by publication of an ANPR is presented in Appendix A[1] to this part.

### § 790.28 Procedures for developing consent agreements and/or test rules for chemicals that have not been designated or recommended with intent to designate by the ITC.

(a) Where EPA believes that testing is needed, it may also develop consent agreements and/or test rules on chemical substances or mixtures that either:

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[1] Editorial Note: Appendix A appears at the end of Subpart E.

(1) Have been recommended but not "recommended with intent to designate" by the ITC.

(2) Have been selected for testing consideration by EPA on its own initiative.

(b) When EPA wishes to initiate negotiations concerning chemicals described in paragraph (a) of this section, it will publish a FEDERAL REGISTER notice describing its tentative evaluation of testing needs, announcing a date for a public course-setting meeting, and inviting persons interested in participating in or monitoring negotiations to contact the Agency in writing. Any negotiations that EPA conducts will conform to the procedures specified in § 790.22(b) and, to the extent feasible, will follow the schedules presented in Appendix A[1] to this part.

(c) EPA will enter into consent agreements on chemicals described in paragraph (a) of this section only if there is a consensus among EPA, affected manufacturers and/or processors, and any other persons who have asked to participate in or monitor negotiations. In determining whether such a consensus exists, EPA will employ the criteria specified in § 790.24. In the absence of consensus, EPA will initiate rulemaking if it concludes that the findings specified in section 4(a) of the Act can be made. The schedule for initiating and completing such rulemaking proceedings will, to the extent feasible, follow the schedule specified in Appendix A[1] to this part.

## Subpart C—Implementation, Enforcement, and Modification of Test Rules

SOURCE: 50 FR 20657, May 17, 1985, unless otherwise noted. Redesignated at 51 FR 23713, June 30, 1986.

### § 790.40 Promulgation of test rules.

(a) If EPA determines that it is necessary to test a chemical substance or mixture by rule under section 4 of the Act, it will promulgate a test rule in Part 799 of this chapter.

(b) EPA will promulgate specific test rules in Part 799 of this chapter either by a single-phase rulemaking procedure or by a two-phase rulemaking procedure.

(1) Under single-phase test rule in Part 799 of this chapter through a notice and comment rulemaking which specifies the following:

(i) Identification of the chemical for which testing is required under the rule.

(ii) The health or environmental effect or effects or other characteristics for which testing is being required.

(iii) Which test substance(s) must be tested.

(iv) Standards for the development of test data.

(v) The EPA Good Laboratory Practice requirements for the required testing.

(vi) Schedule for submission of interim reports and/or final reports to EPA.

(vii) Who must submit either letters of intent to conduct testing or exemption applications.

(viii) What types of data EPA will examine in determining equivalence if more than one test substance is to be tested.

(2) Under two-phase test rule development, EPA will promulgate a Phase I test rule in Part 799 of this chapter through a notice and comment rulemaking which specifies the following:

(i) Identification of the chemical for which testing is required under the rule.

(ii) The health or environmental effect or effects or other characteristics for which testing is being required.

(iii) Which test substance(s) must be tested.

(iv) A reference to appropriate guidelines for the development of test data.

(v) The EPA Good Laboratory Practice requirements for the required testing.

(vi) Who must submit either letters of intent to conduct testing and study plans, or exemption applications.

(vii) What types of data EPA will examine in determining equivalence if more than one test substance is to be tested.

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[1] Editorial Note: Appendix A appears at the end of Subpart E.

(3) Under two-phase test rule development, test standards and schedules will be developed in a second phase of rulemaking as described in §§ 790.50 and 790.52.

[50 FR 20657, May 17, 1985. Redesignated and amended at 51 FR 23713, June 30, 1986]

### § 790.42 Persons subject to a test rule.

(a) Each test rule described in § 790.40 will specify whether manufacturers, processors, or both are subject to the requirement for testing of the subject chemical under section 4(b)(3)(B) of the Act and will indicate who will be required to submit letters of intent to conduct testing.

(1) If testing is being required to allow evaluation of risks:

(i) Primarily associated with manufacture of the chemical, or

(ii) Associated with both manufacturer and processing of the chemical, or

(iii) Associated with distribution in commerce, use, and/or disposal activities concerning the chemical, each manufacturer of the chemical will be subject and must comply with the requirements of the test rule.

(2) While legally subject to the test rule in circumstances described in paragraphs (a)(1) (ii) and (iii) of this section, processors of the chemical must comply with the requirements of the test rule only if processors are directed to do so in a subsequent notice as set forth in § 790.48(b).

(3) If testing is being required to allow evaluation of risks associated solely with processing of the chemical, processors will be subject and must comply with the requirements of the test rule.

(b) [Reserved]

### § 790.45 Submission of letter of intent to conduct testing or exemption application.

(a) No later than 30 days after the effective date of a test rule described in § 790.40, each person subject to that rule and required to comply with the requirements of that rule as provided in § 790.42(a) must, for each test required, either notify EPA by letter of his or her intent to conduct testing or submit to EPA an application for an exemption from testing requirements for the test.

(b) EPA will consider letters of intent to test as commitments to sponsor the tests for which they are submitted unless EPA agrees to the substitution of an exemption application in instances where more than one person indicates an intent to sponsor equivalent tests.

(c) Each letter of intent to conduct testing must include:

(1) Identification of test rule.

(2) Name, address, and telephone number of the firm(s) which will be sponsoring the tests.

(3) Name, address, and telephone number of the appropriate individual to contact for further information.

(4) For sponsors participating in a testing consortium—a list of all members of the consortium, the signature of an authorized representative of each member, and a designation of who is to serve as principal sponsor.

(5) A list of the testing requirements for which the sponsor(s) intends to conduct tests.

(6) If EPA is requiring testing of more than one representative substance—which test substance the sponsor(s) intends to use in each of the tests.

(d)(1) Any person not manufacturing or processing the subject chemical as of the effective date of the test rule describing in § 790.40 or by 30 days after the effective date of the rule who, before the end of the reimbursement period, manufacturers or processes the test chemical and who is subject to and required to comply with the requirements of the test rule must submit the letter of intent to test or an exemption application required by paragraph (a) of this section by the date manufacture or processing begins, or

(2) When both manufacturers and processors are subject to the rule, any person not processing the subject chemical as of the effective date of the test rule described in § 790.40 or by 30 days after publication of the FEDERAL REGISTER notice described in § 790.48(b)(2) who, before the end of the reimbursement period, processes the test chemical and who is required to comply with the requirements of

the rule must submit the letter of intent to test or an exemption application required by § 790.48(b)(3) of the date processing begins.

(e) Manufacturers subject to a test rule described in § 790.40 who do not submit to EPA either a letter of their intent to conduct tests or a request for an exemption from testing for each test for which testing is required in the test rule will be considered in violation of that rule beginning on the 31st day after the effective date of the test rule described in § 790.40 or on the date manufacture begins as described in paragraph (d) of this section.

(f) Processors subject to a test rule described in § 790.40 and required to comply with the requirements of test rule pursuant to § 790.42(a)(2) or a Federal Register notice as described in § 790.48(b)(2) who do not submit to EPA either a letter of their intent to conduct tests or a request for an exemption for each test for which testing is required in the test rule will be considered in violation of that rule beginning on the 31st day after the effective date of the test rule described in § 790.40 or 31 days after publication of the Federal Register notice described in § 790.48(b)(2) or on the date processing begins as described in paragraph (d) of this section, as appropriate.

### § 790.48 Procedure if no one submits a letter of intent to conduct testing.

(a) *If only manufacturers are subject to the rule.* (1) This paragraph applies if testing is being required solely to allow evaluation of risks associated with manufacturing and the test rule described in § 790.40 states that manufacturers only are responsible for testing.

(2) If no manufacturer subject to the test rule has notified EPA of its intent to conduct one or more of the required tests within 30 days after the effective date of the test rule described in § 790.40, EPA will notify all the manufacturers by certified mail or publish a notice in the Federal Register of this fact specifying the tests for which no letter of intent has been submitted and will give the manufacturers an opportunity to take corrective action.

(3) If no manufacturer submits a letter of intent to conduct one or more of the required tests within 30 days after receipt of the certified letter or publication of the Federal Register notice described in paragraph (a)(2) of this section, all manufacturers subject to the rule will be in violation of the test rule from the 31st day after receipt of the certified letter or publication of the Federal Register notice described in this paragraph.

(b) *If manufacturers and processors are subject to the rule.* (1) This paragraph applies if testing is being required to allow evaluation of risks associated with manufacturing and processing or with distribution in commerce, use, or disposal of the chemical and the test rule described in § 790.40 states that manufacturers and processors are responsible for testing.

(2) If no manufacturer subject to the rule has notified EPA of its intent to conduct testing for one or more of the required tests within 30 days after the effective date of the test rule described in § 790.40, EPA will publish a notice in the Federal Register of this fact specifying the tests for which no letter of intent has been submitted.

(3) No later than 30 days after the date of publication of the Federal Register notice described in paragraph (b)(2) of this section, each person processing the subject chemical as of the effective date of the test rule described in § 790.40 or by 30 days after the date of publication of the Federal Register notice described in paragraph (b)(2) of this section must, for each test specified in the Federal Register notice, either notify EPA by letter of his or her intent to conduct testing or submit to EPA an application for an exemption from the testing requirements for the test.

(4) If no manufacturer or processor of the test chemical has submitted a letter of intent to conduct one or more of the required tests within 30 days after the date of publication of the Federal Register notice described in paragraph (b)(2) of this section, EPA will notify all manufacturers and processors by certified letter or publish a Federal Register notice of this fact specifying the tests for which no letter of intent has been submitted. This

letter or Federal Register notice will give the manufacturers and processors an opportunity to take corrective action.

(5) If no manufacturer or processor submits a letter of intent to conduct one or more of the required tests within 30 days after receipt of the certified letter or publication of the Federal Register notice described in paragraph (b)(4) of this section, all manufacturers and processors subject to the rule will be in violation of the test rule from the 31st day after receipt of the certified letter or publication of the Federal Register notice described in paragraph (b)(4) of this section.

(c) *Only processors are subject to the rule.* (1) This paragraph applies if testing is being required solely to allow evaluation of risks associated with processing and the test rule described in § 790.40 states that only processors are responsible for testing.

(2) If no processor subject to the rule has notified EPA of its intent to conduct one or more of the required tests within 30 days after the effective date of the test rule described in § 790.40, EPA will notify all the processors by certified mail or publish a notice in the Federal Register of this fact, specifying the tests for which no letter of intent has been submitted and give the processors an opportunity to take corrective action.

(3) If no processor submits a letter of intent to conduct one or more of the required tests within 30 days after receipt of the certified letter or publication of the Federal Register notice described in paragraph (c)(2) of this section, all processors subject to the rule will be in violation of the test rule from the 31st day after receipt of the certified letter or publication of the Federal Register notice described in this paragraph.

### § 790.50 Submission of study plans.

(a) *Who must submit study plans.* (1) Persons who notify EPA of their intent to conduct tests in compliance with the requirements of a single-phase rule as described in § 790.40(b)(1) must submit study plans for those tests no later than 45 days before the initiation of each of these tests.

(2) Persons who notify EPA of their intent to conduct tests in compliance with the requirements of a Phase I test rule as described in § 790.40(b)(2) must submit the following:

(i) Proposed study plans for those tests must be submitted on or before 90 days after the effective date of the Phase I rule; or, for processors complying with the notice described in § 790.40(b)(2), 90 days after the publication date of that notice; or 60 days after the date manufacture or processing begins as described in § 790.45(d), as appropriate; and

(ii) [Reserved]

(3) Study plans must be prepared according to the requirements of this Subpart B and Part 792 of this chapter. Only one set of study plans should be prepared and submitted by persons who are jointly sponsoring testing.

(4) Any person subject to a test rule may submit a study plan for any test required by the rule at any time, regardless of whether the person previously submitted an application for exemption from testing for that test.

(5) Unless EPA has granted an extension of time for submission of proposed study plans, manufacturers who notify EPA that they intend to conduct testing in compliance with the requirements of a Phase I test rule as described in § 790.40(b)(2) and who do not submit proposed study plans for those tests on or before 90 days after the effective date of the Phase I test rule or 60 days after the date manufacture begins as described in § 790.45(d) will be considered in violation of the test rule as if no letter of intent to test had been submitted.

(6) Unless EPA has granted an extension of time for submission of proposed study plans, processors who notify EPA that they intend to conduct testing in compliance with the requirements of a Phase I test rule as described in § 790.40(b)(2) and who do not submit proposed study plans for those tests on or before 90 days after the effective date of the Phase I test rule or 90 days after the publication date of the notice described in § 790.48(b)(2), or 60 days after the date processing begins as described in

§ 790.45(d), as appropriate, will be considered in violation of the test rule as if no letter of intent to test had been submitted.

(b) *Extensions of time for submission of study plans.* (1) The Agency may grant requests for additional time for the development of study plans on a case-by-case basis. Requests for additional time for study plan development must be made in writing to EPA. Each extension request must demonstrate why that extension should be granted.

(2) Under two-phase rulemaking, extension requests must be submitted to EPA within 60 days after the effective date of the Phase I test rule as described in § 790.40(b)(2); or for processors complying with the notice described in § 790.48(b)(2), 60 days after the publication date of that notice; or 30 days after the date manufacture or processing begins as described in § 790.45(d), as appropriate.

(3) EPA will notify the submitter by certified mail of EPA's decision to grant or deny an extension request.

(4) Persons who have been granted an extension of time for submission of study plans as described in paragraph (b)(1) of this section and who do not submit proposed study plans in compliance with the requirements of a Phase I test rule in accordance with the new deadline granted by EPA will be considered in violation of the test rule as if no letter of intent to test had been submitted as described in § 790.45(e) and (f).

(c) *Content of study plans.* (1) All study plans are required to contain the following information:

(i) Identity of the test rule.

(ii) The specific test requirements of that rule to be covered by the study plan.

(iii)(A) The names and addresses of the test sponsors.

(B) The names, addresses, and telephone numbers of the responsible administrative officials and project manager(s) in the principal sponsor's organization.

(C) The name, address, and telephone number of the appropriate individual to contact for oral and written communications with EPA.

(D) (*1*) The names and addresses of the testing facilities and the names, addresses, and telephone numbers of the testing facilities' administrative officials and project manager(s) responsible for the testing.

(*2*) Brief summaries of the training and experience of each professional involved in the study, including study director, veterinarian(s), toxicologist(s), pathologist(s), chemist(s), microbiologist(s), and laboratory assistants.

(iv) Identity and data on the chemical substance(s) being tested, including physical constants, spectral data, chemical analysis, and stability under test and storage conditions, as appropriate.

(v) Study protocol, including rationale for species/strain selection; dose selection (and supporting data); route(s) or method(s) of exposure; description of diet to be used and its source, including nutrients and contaminants and their concentrations; for *in vitro* test systems, a description of culture medium and its source; and a summary of expected spontaneous chronic diseases (including tumors), genealogy, and life span.

(vi) Schedule for initiation and completion of each short-term test and of each major phase of long-term tests; schedule for submission of interim progress and final reports to EPA.

(2) Information required in paragraph (c)(1)(iii)(D) of this section is not required in proposed study plans submitted in compliance with the requirements of a Phase I test rule if the information is not available at the time of study plan submission; however, the information must be submitted before the initiation of testing.

(d) *Incomplete study plans.* (1) Upon receipt of a study plan, EPA will review the study plan to determine whether it complies with paragraph (c) of this section. If EPA determines that the study plan does not comply with paragraph (c) of this section, EPA will notify the submitter that the submission is incomplete and will identify the deficiencies and the steps necessary to complete the submission.

(2) The submitter will have 15 days after the day it receives this notice to

submit appropriate information to make the study plan complete.

(3) If the submitter fails to provide appropriate information to complete a proposed study plan submitted in compliance with the requirements of a Phase I test rule on or before 15 days after receipt of the notice, the submitter will be considered in violation of the test rule as if no letter of intent to conduct the test had been submitted as described in § 790.45(e) and (f).

(Approved by the Office of Management and Budget under control number 2070-0033)

[50 FR 20657, May 17, 1985. Redesignated and amended at 51 FR 23713, June 30, 1986. Further amended at 52 FR 36569, Sept. 30, 1987]

### § 790.52 Phase II test rule.

(a) If EPA determines that the proposed study plan described in § 790.50(a)(2) complies with § 790.50(c), EPA will publish a proposed Phase II test rule in the FEDERAL REGISTER requesting comments on the ability of the proposed study plan to ensure that data from the test will be reliable and adequate.

(b) EPA will provide a 45-day comment period and will provide an opportunity for an oral presentation upon the request of any person. EPA may extend the comment period if it appears from the nature of the issues raised by EPA's review or from public comments that further comment is warranted.

(c) After receiving and considering public comments on the study plan, EPA will adopt, as proposed or as modified in response to EPA review and public comments, the study protocol section of the study plan, as defined by § 790.50(c)(1)(v) of this chapter, as the test standard for the required testing, and the schedule section of the study plan, as defined by § 790.50(c)(1)(vi) of this chapter, as the schedule for the required testing in a final Phase II test rule.

[50 FR 20657, May 17, 1985. Redesignated at 51 FR 23713, June 30, 1986, and amended at 52 FR 36569, Sept. 30, 1987]

### § 790.55 Modification of test standards or schedules during conduct of test.

(a) *Application.* Any test sponsor who wishes to modify the test standards or schedules for any test required under a test rule must submit an application in accordance with this paragraph. Application for modification must be made in writing to the Director, Office of Compliance Monitoring (EN-342), Office of Pesticides and Toxic Substances, EPA, 401 M St., SW., Washington, DC 20460, or by phone, with written confirmation to follow within 10 working days. Applications must include appropriate explanation of why the modification is necessary.

(b) *Adoption.* (1) Where EPA concludes that the requested modification of a test standard or schedule for a test required under a test rule is appropriate, EPA will proceed in accordance with this paragraph (b).

(2) Where, in EPA's judgment, the requested modification of the test standard or schedule would not alter the scope of the test or significantly change the schedule for completing the test, EPA will not ask for public comment before approving the modification. EPA will notify the test sponsor by letter of EPA's approval. EPA will place copies of each application and EPA approval letter in the rulemaking record for the test rule in question. EPA will publish a notice annually in the FEDERAL REGISTER indicating the test standards or schedules for tests required in test rules which have been modified under this paragraph (b)(2) and describing the nature of the modifications. Until the FEDERAL REGISTER notice is published, any modification approved by EPA under this paragraph (b)(2) shall apply only to the test sponsor who applied for the modification under this paragraph (a) of this section.

(3) Where, in EPA's judgment, the requested modification of a test standard or schedule would alter the scope of the test or significantly change the schedule for completing the test, EPA will publish a notice in the FEDERAL REGISTER requesting comment on the proposed modification. However, EPA will approve a requested modification

of a test standard under this paragraph (b)(3) without first seeking public comment if EPA believes that an immediate modification to the test standard is necessary to preserve the accuracy or validity of an ongoing test. EPA will publish a notice in the FEDERAL REGISTER approving any modification under this paragraph (b)(3).

(4) For purposes of this paragraph (b), a requested modification of a test standard or schedule for a test required under a test rule would alter the scope of the test or significantly change the schedule for completing the test if the modification would:

(i) Change the test species.

(ii) Change the route of administration of the test chemical.

(iii) Change the period of time during which the test species is exposed to the test chemical.

(iv) Result in a delay of over six months in receiving the final report of the test.

(c) *Disapproval.* Where EPA concludes that the requested modification of a test standard or schedule for a test required under a test rule is not appropriate, EPA will so notify the test sponsor in writing.

[50 FR 20657, May 17, 1985. Redesignated at 51 FR 23713, June 30, 1986, and amended at 52 FR 36571, Sept. 30, 1987]

### § 790.59 Failure to comply with a test rule.

(a) Persons who notified EPA of their intent to conduct a test required in a test rule in Part 799 of this chapter and who fail to conduct the test in accordance with the test standards and schedules adopted in the test rule, or as modified in accordance with § 790.55, will be in violation of the rule.

(b) Any person who fails or refuses to comply with any aspect of this Part or a test rule under Part 799 of this chapter is in violation of section 15 of the Act. EPA will treat violations of the Good Laboratory Practice standards as indicated in § 792.17 of this chapter.

## Subpart D—Implementation, Enforcement and Modification of Consent Agreements

SOURCE: 51 FR 23715, June 30, 1986, unless otherwise noted.

### § 790.60 Contents of consent agreements.

(a) *Standard provisions.* All consent agreements will contain the following provisions:

(1) Identification of the chemical(s) to be tested.

(2) The health effects, environmental effects and/or other characteristics for which testing will be required.

(3) The names and addresses of each manufacturer and/or processor who will sign the agreement.

(4) The name and address of the manufacturer, processor or other entity who has agreed to act as the principal test sponsor.

(5) The technical or commercial grade, level of purity or other characteristics of the test substances(s) or mixture(s).

(6) Standards for the development of test data.

(7) A requirement that testing will be conducted in accordance with the EPA Good Laboratory Practice (GLP) regulations (40 CFR Part 792).

(8) Schedules with reasonable timetables and deadlines for initiating testing and submitting interim progress and/or final reports to EPA.

(9) A requirement that the principal sponsor will submit a study plan to EPA in accordance with § 790.62.

(10) A statement that the results of testing conducted pursuant to the consent agreement will be announced to the public in accordance with the procedures specified in section 4(d) of the Act and that the disclosure of data generated by such testing will be governed by section 14(b) of the Act.

(11) A requirement that the manufacturers and/or processors signing the consent agreement will comply with the notification requirements of section 12(b)(1) of the Act and Part 707 of this chapter if they export or intend to export the substance or mixture for which the submission of data is required under the agreement and a statement that any other person who

exports or intends to export such substance or mixture is subject to the above cited export notification requirements.

(12) A requirement that, in the event EPA promulgates a significant new use rule applicable to the test chemical under section 5(a)(2), the consent agreement will have the status of a test rule for purposes of section 5(b)(1)(A) and manufacturers and/or processors signing the agreement will comply with the data submission requirements imposed by that provision.

(13) A statement that each manufacturer and/or processor signing the agreement agrees that violation of its requirements will constitute a "prohibited act" under section 15(1) of the Act and will trigger all provisions of TSCA applicable to a violation of section 15.

(14) A statement that, in the event one or more provisions of the agreement are determined to be unenforceable by a court, the remainder of the agreement would not be presumed to be valid and EPA will then either initiate a rulemaking proceeding or publish in the FEDERAL REGISTER the Administrator's reason for not initiating such a proceeding.

(15) A statement that the Agency may conduct laboratory inspections and/or study audits of the testing being conducted pursuant to the consent agreement in accordance with the authority and procedures contained in section 11 of the Act.

(16) A statement that EPA acceptance of a consent agreement constitutes "final agency action" for purposes of 5 U.S.C. 704.

(17) Any other requirements that the parties agree are necessary to achieve the purposes of the Act.

(b) *Contents of standards for the development of data.* The standards for the development of the data included in consent agreements will be based on the TSCA test guidelines in 40 CFR Parts 796, 797, and 798, the Organization for Economic Cooperation and Development (OECD) test guidelines, the EPA pesticide assessment guidelines published by The National Technical Information Service (NTIS), or other suitable test methodologies. During the negotiation of consent agreements, EPA will initially propose suitable test guidelines as the required test standards; manufacturers and processors or other interested parties may then suggest alternative methodologies or modifications to the Agency's proposed guidelines. These alternative methodologies or modifications will be adopted only where, in the judgment of EPA, they will develop at least equally reliable and adequate data on the chemical substance or mixture subject to the agreement.

(c) *Statement of rationale for consent agreement.* EPA will prepare a written explanation of the basis for each consent agreement. This document will summarize the agreement, describe any ITC testing recommendations for the chemical involved, outline the chemical's use and exposure characteristics, and explain the objectives of the testing to be conducted and the rationale for the specific studies selected. This document will be published in the FEDERAL REGISTER and, for ITC-designated chemicals, will constitute the statement of EPA's reasons for not initiating rulemaking required by section 4(e)(1)(B) of the Act.

### § 790.62 Submission of study plans and conduct of testing.

(a) *Timing of submission.* The principal sponsor of testing conducted pursuant to a consent agreement shall submit a study plan no later than 45 days prior to the initiation of testing. The Agency may grant requests for additional time to submit study plans on a case-by-case basis. Requests for additional time for study plan development must be made in writing to the Director, Office of Compliance Monitoring (EN-342), Office of Pesticides and Toxic Substances, EPA, 401 M Street, SW., Washington, DC 20460. Each extension request must demonstrate why the extension should be granted. EPA will notify the submitter by certified mail of the Agency's decision to grant or deny any extension request. Extensions of time for submission of study plans granted by EPA will not relieve manufacturers and/or processors of the obligation to meet the consent agreement's schedule for initiating testing and submitting interim and/or final reports.

(b) *Content of study plans.* All study plans are required to contain the following information:

(1) Identity of the consent agreement under which testing will be performed.

(2) The specific test requirements to be covered by the study plan.

(3) The name and address of the principal test sponsor.

(4) The names, addresses, and telephone numbers of the responsible administrative official[s] and project manager[s] in the principal sponsor's organization.

(5) The names, addresses, and telephone numbers of the technical contacts at each manufacturer and/or processor subject to the agreement.

(6) The names and addresses of the testing facilities responsible for the testing and the names, addresses, and telephone numbers of the administrative officials[s] and project manager[s] assigned to oversee the testing program at these facilities.

(7) Brief summaries of the training and experience of each professional involved in the study, including study director, veterinarian[s], toxicologist[s], pathologist[s], chemist[s], microbiologist[s], and laboratory assistants.

(8) Identity and supporting data on the chemical substance[s] being tested, including physical constants, spectral data, chemical analysis, and stability under test and storage conditions, as appropriate.

(9) Study protocol, including rationale for species/strain/sex selection; dose selection (and supporting data); route[s] or method[s] and form[s] and duration of exposure; description of diet to be used and its source, including nutrients and contaminants and their concentrations; description of the controls; for *in vitro* test systems, a description of culture medium and its source; and a summary of expected spontaneous chronic diseases (including tumors), genealogy, and life span.

(10) A schedule, with reasonable timeables and deadlines, for initiation and completion of each short-term test and of each major phases of long-term tests, and submission of interim progress and/or final reports to EPA.

(c) *Review and modification.* (1) Upon receipt of a study plan, EPA will review it to determine whether it complies with paragraph (b) of this section and the consent agreement. If EPA determines that the study plan does not comply with paragraph (b) of this section, EPA will notify the submitter that the submission is incomplete and will identify the deficiencies and the steps necessary to complete the submission.

(2) The submitter will have 15 days after the day it receives a notice under paragraph (c)(1) of this section to submit appropriate information to make the study plan complete.

(3) If the submitter fails to provide appropriate information to complete a study plan within 15 days after having received a notice under paragraph (c)(1) of this section, the submitter will be considered to be in violation of the consent agreement and subject to enforcement proceedings pursuant to § 790.65 (c) and (d).

(d) *Functions of the principal test sponsor.* When testing is being conducted pursuant to a consent agreement, the principal test sponsor will be responsible for submitting interim progress and final reports to EPA, informing the Agency of any proposed changes in standards for the development of data, study plans or testing schedules, and communicating with the Agency about laboratory inspections and other matters affecting the progress of testing.

### § 790.65 Failure to comply with a consent agreement.

(a) Manufacturers and/or processors who have signed a consent agreement and who fail to comply with the test requirements, test standards, GLP regulations, schedules, or other provisions contained in the consent agreement, or in modifications to the agreement adopted pursuant to § 790.68, will be in violation of the consent agreement.

(b) The Agency considers failure to comply with any aspect of a consent agreement to be a "prohibited act" under section 15 of TSCA, subject to all of the provisions of the Act applicable to violations of section 15. Section 15(1) of TSCA makes it unlawful for any person to fail or refuse to comply with any rule or order issued under section 4. Consent agreements adopted pursuant to this part are "orders issued under section 4" for purposes of section 15(1) of TSCA.

(c) Manufacturers and/or processors who violate consent agreements are subject to criminal and/or civil liability. Under the penalty provisions of section 16 of TSCA, such firms could be subject to a civil penalty of up to $25,000 per violation with each day in violation constituting a separate violation of section 15. Intentional violations could lead to the imposition of criminal penalties of up to $25,000 for each day of violation and imprisonment for up to one year. In addition, EPA could invoke the remedies available under section 17 of TSCA, including seeking an injunction to compel adherence to the requirements of the consent agreement.

(d) Noncompliance with a consent agreement will constitute conduct "in violation of this Act" under section 20(a)(1) of TSCA. Thus, failure to comply with the requirements of a consent agreement could result in a citizens' civil action under section 20(a)(1) of TSCA.

### § 790.68 Modification of consent agreements.

(a) *Changes in the scope of testing.* (1) Manufacturers or processors subject to a consent agreement, other persons or EPA may seek modifications in the scope of testing performed under the consent agreement. If, upon receiving a request for modification, EPA determines that new issues have been raised that warrant reconsideration of the scope of testing, or if EPA determines on its own that such reconsideration is appropriate, EPA will publish a FEDERAL REGISTER notice describing the proposed modification and soliciting public comment. If, based on the comments received, EPA concludes that differences of opinion may exist about the proposed modification, EPA will establish a schedule for conducting negotiations and invite parties who wish to participate in or monitor these negotiations to contact the Agency in writing. Any negotiations that EPA conducts will conform to the procedures specified in § 790.22(b).

(2) The scope of testing required by a consent agreement will be modified only where there is a consensus concerning the modified testing requirements among EPA, affected manufacturers and/or processors, and other persons who have asked to participate in or monitor negotiations under paragraph (a)(1) of this section. In determining whether a consensus exists, EPA will employ the criteria specified in § 790.24. In the absence of consensus, EPA may initiate rulemaking under section 4(a) of the Act if it concludes that any testing beyond that required by the consent agreement is necessary and that the other statutory findings required by section 4(a) can be made. While such rulemaking proceedings are underway, the consent agreement will remain in effect unless EPA finds that the testing required by the agreement is or may be unnecessary in view of the testing requirements included in EPA's proposed rule.

(b) *Changes in test standards or schedules.* (1) Any test sponsor who wishes to modify the test standards or schedules for any test required under a consent agreement must submit an application in accordance with this subsection. Applications for modification must be made in writing to the Director, Office of Compliance Monitoring (EN-342), Office of Pesticides and Toxic Substances, EPA, 401 M Street, SW., Washington, DC 20460, or by phone, with written confirmation to follow within 10 working days. Applications must include an appropriate explanation of why the modification is necessary.

(2)(i) Where EPA concludes that the requested modification of a test standard or schedule for a test required under a consent agreement is appropriate, EPA will proceed in accordance with this paragraph (b)(2).

(ii) Where, in EPA's judgment, the requested modification of a test standard or schedule would not alter the scope of the test or significantly change the schedule for completing the test, EPA will not ask for public comment before approving the modification. EPA will notify the test sponsor, and any other persons who have signed the consent agreement, by letter of EPA's approval. EPA will place copies of each application and EPA approval letter in the administrative record maintained for the consent agreement in question. EPA will publish a notice annually in the FEDERAL REGISTER indicating the test standards or schedules for test required in consent agreements which have been modified under this paragraph (b)(2)(ii) and describing the nature of the modifications.

(iii) Where, in EPA's judgment, the requested modification of a test standard or schedule would alter the scope of the test or significantly change the schedule for completing the test, EPA will publish a notice in the FEDERAL REGISTER requesting comment on the proposed modification. However, EPA will approval a requested modification of a test standard under this paragraph (b)(2)(iii) without first seeking public comment if EPA believes that an immediate modification to the test standard is necessary to preserve the accuracy or validity of an ongoing test. EPA will publish a notice in the FEDERAL REGISTER approving any modification under this paragraph (b)(2)(iii).

(iv) For purposes of this paragraph (b)(2), a requested modification of a test standard of schedule for a test required under a consent agreement would alter the scope of the test or significantly change the schedule for completing the test if the modification would:

(A) Change the test species.

(B) Change the route of administration of the test chemical.

(C) Change the period of time during which the test species is exposed to the test chemical.

(D) Result in a delay of over six months in receiving final report of the test.

(3) Where EPA concludes that the requested modification of a test standard or schedule for a test requirement under a consent agreement is not appropriate, EPA will so notify the test sponsor in writing.

[51 FR 23715, June 30, 1986, as amended at 52 FR 36571, Sept. 30, 1987]

## Subpart E—Exemptions From Test Rules

SOURCE: 50 FR 20660, May 17, 1985, unless otherwise noted.

### § 790.80 Submission of exemption applications.

(a) *Who should file applications.* (1) Any manufacturer or processor subject to a test rule in Part 799 of this chapter may submit an application to EPA for an exemption from performing any or all of the tests required under the test rule.

(2) Processors will not be required to apply for an exemption or conduct testing unless EPA so specifies in a test rule or in a special FEDERAL REGISTER notice as described in § 790.48(b)(2) under the following circumstances:

(i) If testing is being required to allow evaluation of risks associated with manufacturing and processing or with distribution in commerce, use, or disposal of the chemical and manufacturers do not submit notice(s) of intent to conduct the required testing; or

(ii) If testing is being required solely to allow evaluation of risks associated with processing of the chemical.

(b) *When applications must be filed.* (1) Exemption applications must be filed within 30 days after the effective date of the test rule described in § 790.40 or, if being submitted in compliance with the FEDERAL REGISTER notice described in § 790.48(b)(2), within 30 days after the publication of that notice.

(2) Exemption applications must be filed by the date manufacture or processing begins by any person not manufacturing or processing the subject chemical as of the effective date of the test rule described in § 790.40 or by 30 days after the effective date of the test rule described in § 790.40, who, before the end of the reimbursement period, manufactures or processes the test substance and who is subject to the requirement to submit either a letter of intent to test or an exemption application.

(3) When both manufacturers and processors are subject to the rule, ex-

emption applications must be filed by the date processing begins by any person not processing as of the effective date of the test rule described in § 790.40 or by 30 days after publication of the FEDERAL REGISTER notice described in § 790.48(b)(2) who, before the end of the reimbursement period, processes the test substance and who is subject to the requirement to submit either a letter of intent to test or an exemption application.

(c) *Scope of application.* A person may apply for an exemption from all, or one or more, specific testing requirements in a test rule in Part 799 of this chapter.

(Approved by the Office of Management and Budget under control number 2070-0033)

### § 790.82 Content of exemption application.

The exemption application must contain:

(a) The identity of the test rule.

(b) The specific testing requirement(s) from which an exemption is sought and the basis for the exemption request.

(c) Name, address, and telephone number of applicant.

(d) Name, address, and telephone number of appropriate individual to contact for further information.

(e)(1) If required in the test rule to establish equivalence:

(i) The chemical identity of the test substance on which the application is based.

(ii) Equivalence data specified in § 790.85.

(2) If a test rule requires testing of a single representative substance, EPA will consider all forms of the chemical subject to that rule to be equivalent and will not require the submission of equivalence data as described in § 790.85.

### § 790.85 Submission of equivalence data.

If EPA requires in a test rule promulgated under section 4 of the Act the testing of two or more test substances which are forms of the same chemical, each exemption applicant must submit the following data:

(a) The chemical identity of each technical-grade chemical substance or mixture manufactured and/or processed by the applicant for which the exemption is sought. The exact type of identifying data required will be specified in the test rule, but may include all characteristics and properties of the applicant's substance or mixture, such as boiling point, melting point, chemical analysis (including identification and amount of impurities), additives, spectral data, and other physical or chemical information that may be relevant in determining whether the applicant's substance or mixture is equivalent to the specific test substance.

(b) The basis for the applicant's belief that the substance or mixture is equivalent to the test substance or mixture.

(c) Any other data which exemption applicants are directed to submit in the test rule which may bear on a determination of equivalence. This may include a description of the process by which each technical-grade chemical substance or mixture for which an exemption is sought is manufactured or processed prior to use or distribution in commerce by the applicant.

### § 790.87 Approval of exemption applications.

(a) EPA will conditionally approve exemption applications if:

(1)(i) For single-phase test rules, EPA has received a letter of intent to conduct the testing from which exemption is sought;

(ii) For two-phase test rules, EPA has received a complete proposed study plan for the testing from which exemption is sought and has adopted the study plan, as proposed or modified, as test standards and schedules in a final Phase II test rule; and

(2) The chemical substance or mixture with respect to which the application was submitted is equivalent to a test substance or mixture for which the required data have been or are being submitted in accordance with a test rule; and

(3) Submission of the required test data concerning that chemical substance or mixture would be duplicative of data which have been or are being submitted to EPA in accordance with a test rule.

(b)(1) If a single representative substance is to be tested under a test rule, EPA will consider all forms of the chemical subject to that rule to be equivalent and will contact the exemption applicant only if information is missing or unclear.

(2) If two or more representative substances are to be tested under a test rule, EPA will evaluate equivalence claims made in each exemption application according to the criteria discussed in the test rule.

(i) If EPA finds an equivalence claim to be in error or inadequately supported, the applicant will be notified by certified mail. The applicant will be given 15 days to provide clarifying information.

(ii) Exemption applicants will be notified that equivalence has been accepted or rejected.

(c) The final Phase II test rule which adopts the study plans in two-phase rulemaking, a separate FEDERAL REGISTER notice in single-phase rulemaking, or a letter by certified mail will give exemption applicants final notice that they have received a conditional exemption. All conditional exemptions thus granted are contingent upon the test sponsors' successful completion of testing according to the specifications in the test rule.

### § 790.88 Denial of exemption application.

(a) EPA may deny any exemption application if:

(1) EPA determines that the applicant has failed to demonstrate that the applicant's chemical is equivalent to the test substance; or

(2) The exemption applicant fails to submit any of the information specified in § 790.82; or

(3) The exemption applicant fails to submit any of the information specified in § 790.85 if required in the test rule; or

(4)(i) For single-phase test rules, EPA has not received a letter of intent to conduct the test for which exemption is sought; or

(ii) For two-phase test rules, EPA has not received an adequate study plan for the test for which exemption is sought; or

(5) The study sponsor(s) fails to initiate the required testing by the deadlines adopted in the test rule; or

(6) The study sponsor(s) fails to submit data as required in the test standard and deadlines for submission of test data as adopted in the test rule or as modified in accordance with § 790.55.

(b) EPA will notify the exemption applicant by certified mail or FEDERAL REGISTER notice of EPA's determination that the exemption application is denied.

### § 790.90 Appeal of denial of exemption application.

(a) Within 30 days after receipt of notification that EPA has denied an application for exemption, the applicant may file an appeal with EPA.

(b) The appeal shall indicate the basis for the applicant's request for reconsideration.

(c)(1) The applicant may also include a request for a hearing. Hearings will be held according to the procedures described in § 790.97.

(2) Hearing requests must be in writing and must be received by EPA within 30 days of receipt of the letter or publication of the FEDERAL REGISTER notice described in § 790.88(b). Hearing requests must provide reasons why a hearing is necessary.

(d) If EPA determines that there are material issues of fact, then the request for a hearing will be granted. If EPA denies a hearing request, EPA will base its decision on the written submission.

(e) EPA will notify the applicant of its decision within 60 days after EPA receives the appeal described in paragraph (a) of this section or within 60 days after completion of a hearing described in paragraph (c) of this section.

(f) The filing of an appeal from the denial of an exemption shall not act to stay the applicant's legal obligations under a test rule promulgated under section 4 of the Act.

### § 790.93 Termination of conditional exemption.

(a) EPA shall terminate a conditional exemption if it determines that:

(1) The test which provided the basis for approval of the exemption application has not been started by the deadlines for initiation of testing adopted in the test rule or modified in accordance with § 790.55; or

(2) Data required by the test rule have not been generated in accordance with the test standards or submitted in accordance with the deadlines for submission of test data that were adopted in the test rule or modified in accordance with § 790.55; or

(3) The testing has not been conducted or the data have not been generated in accordance with the Good Laboratory Practice requirements in Part 792 of this chapter.

(b) If EPA determines that one or more of the criteria listed in paragraph (a) of this section has been met, EPA will notify each holder of an affected conditional exemption by certified mail or FEDERAL REGISTER notice of EPA's intent to terminate that conditional exemption.

(c) Within 30 days after receipt of a letter of notification or publication of a notice in the FEDERAL REGISTER that EPA intends to terminate a conditional exemption, the exemption holder may submit information to rebut EPA's preliminary decision or notify EPA by letter of its intent to conduct the required test pursuant to the test standard established in the final test rule. Such a letter of intent shall contain all of the information required by § 790.45(c).

(d)(1) The exemption holder may also include a request for a hearing. Hearings will be held in accordance with the procedures set forth in § 790.97.

(2) Hearing requests must be in writing and must be received by EPA within 30 days after receipt of the letter or publication in the FEDERAL REGISTER notice described in paragraph (b) of this section.

(e) EPA will notify the exemption holder by certified letter or by FEDERAL REGISTER notice of EPA's final decision concerning termination of conditional exemptions and will give instructions as to what actions the former exemption holder must take to avoid being found in violation of the test rule.

### § 790.97 Hearing procedures.

(a) Hearing requests must be in writing to EPA and must include the applicant's basis for appealing EPA's decision.

(b) If more than one applicant has requested a hearing on similar grounds, all of those appeals will be considered at the same hearing unless confidentiality claims preclude a joint hearing.

(c) EPA will notify each applicant of EPA's decision within 60 days after the hearing.

### § 790.99 Statement of financial responsibility.

Each applicant for an exemption shall submit the following sworn statement with his or her application:

> I understand that if this application is granted before the reimbursement period described in section 4(c)(3)(B) of TSCA expires, I must pay fair and equitable reimbursement to the person or persons who incurred or shared in the costs of complying with the requirement to submit data and upon whose data the granting of my application was based.

### APPENDIX A TO SUBPART E—SCHEDULE FOR DEVELOPING CONSENT AGREEMENTS AND TEST RULES

EPA intends to follow the schedule set forth in this Appendix to evaluate testing candidates, conduct negotiations, develop consent agreements where appropriate, and propose and promulgate test rules in those instances where testing can be required under section 4(a) of TSCA but agreement cannot be reached in timely manner on a consent agreement. Where deadlines are imposed by the statute, they are binding on EPA and will be observed by the Agency. The remaining dates represent targets that EPA intends to meet.

This schedule is based on what EPA currently believes are reasonable target dates. As EPA gains experience with the process and determines the feasibility of these schedules, it may adjust the schedule accordingly. EPA will solicit public comment before implementing any changes in the schedule.

| Week [1] | Event |
|---|---|
| 0 | Receive ITC report, recommendation. |
| 2 | Publish ITC report, 8(a) and 8(d) notices, and invitation for public participation in negotiations. |

| Week [1] | Event |
|---|---|
| 3-6 | Comment period on ITC report. |
| 6 | Public focus meeting. |
| 7-14 | 8(a) and 8(d) reporting period. |
| 22 | Public meeting on course-setting decision and deadline for requests to participate in negotiations. |
| 22-30 | Negotiations. |
| 32 | EPA decision point: consent agreement or test rule. |

[1] The dates contained in the left-hand column are calculated from the date EPA receives the ITC report recommending a chemical for testing.

| Week | Consent Agreement | Week | Test Rule |
|---|---|---|---|
| 36-40 | Comment period on consent agreement | 32-60 | Rule preparation, agency review and sign-off. |
| 42 | Comment resolution meeting if necessary | 62 | Publish proposed rule in FEDERAL REGISTER.[1] |
| 46 | Sign-off consent agreement and FEDERAL REGISTER notice. | 70-106 | Agency reviews comments; preparation of final rule or no-test decision, agency review and sign-off.[1] |
| 50 | Publish FEDERAL REGISTER notice | 108 | Publish final rule or no-test decision in FEDERAL REGISTER.[1] |

[1] As stated in § 790.26, EPA may publish an Advance Notice of Proposed Rulemaking (ANPR) where the testing recommendations of the ITC raise unusually novel and complex issues that require additional Agency review and opportunity for public comment. EPA intends to publish such ANPRs by Week 62 following receipt of the initial ITC report; to publish a proposed rule or decision-not-to-test by Week 108; and to publish a final rule or notice terminating the rulemaking process by Week 154.

[51 FR 23717, June 30, 1986]

# PART 791—DATA REIMBURSEMENT

### Subpart A—General Provisions

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791.105 Prohibited acts.

AUTHORITY: 15 U.S.C. 2603 and 2607.

SOURCE: 48 FR 31791, July 11, 1983, unless otherwise noted.

## Subpart A—General Provisions

### § 791.1 Scope and authority.

(a) This part establishes procedures and criteria to be used in determining fair amounts of reimbursement for testing costs incurred under section 4(a) of the Toxic Substances Control Act (TSCA) (15 U.S.C. 2603(a)).

(b) Section 4(c) of TSCA requires EPA to develop rules for the determination of fair and equitable reimbursement (15 U.S.C. 2603 (c)).

### § 791.2 Applicability.

(a) This rule is potentially applicable to all manufacturers, importers and processors who may be required by a specific test rule promulgated under section 4(a) of TSCA to conduct tests and submit data, and who seek the assistance of the Administrator in determining the amount or method of reimbursement. Persons subject to a test rule have an obligation from the date the test rule becomes effective until the end of the reimbursement period, either to test or to obtain an exemption and pay reimbursement.

(b) The provisions of this rule will take effect only when private efforts to resolve a dispute have failed and a manufacturer or processor requests EPA's assistance.

### § 791.3 Definitions.

Terms defined in the Act, and not explicitly defined herein, are used with the meanings given in the Act.

(a) "The Act" refers to the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601 *et seq.*).

(b) "The Agency" or "EPA" refers to the Environmental Protection Agency.

(c) "Byproduct" refers to a chemical substance produced without a separate commercial intent during the manufacture, processing, use or disposal of another chemical substance or mixture.

(d) "Dispute" refers to a present controversy between parties subject to a test rule over the amount or method of reimbursement for the cost of developing health and environmental data on the test chemical.

(e) "Exemption holder" refers to a manufacturer or processor, subject to a test rule, that has received an exemption under sections 4(c)(1) or 4(c)(2) of TSCA from the requirement to conduct a test and submit data.

(f) "Impurity" refers to a chemical substance unintentionally present with another chemical substance or mixture.

(g) A "party" refers to a person subject to a section 4 test rule, who:

(1) Seeks reimbursement from another person under these rules, or

(2) From whom reimbursement is sought under these rules.

(h) "Reimbursement period" refers to a period that begins when the data from the last non-duplicative test to be completed under a test rule is submitted to EPA and ends after an amount of time equal to that which had been required to develop that data or after 5 years, whichever is later.

(i) "Small business" refers to a manufacturer or importer whose annual sales, when combined with those of its parent company (if any) are less than $30 million.

(j) "Test rule" refers to a regulation ordering the development of data on health or environmental effects or chemical fate for a chemical substance or mixture pursuant to TSCA section 4(a).

## Subpart B—Hearing Procedures

### § 791.20 Initiation of reimbursement proceeding.

(a) When persons subject to a test rule are unable to reach an agreement on the amount or method of reimbursement for test data development as described in TSCA section 4(c)(3)(A), any of them may initiate a proceeding by filing two signed copies of a request for a hearing with a regional office of the American Arbitration Association and mailing a copy of the request to EPA, and to each person from whom they seek reimbursement, or who seeks reimbursement from them.

(b) The request for hearing must contain the following:

(1) The names and addresses of the filing party and its counsel, if any.

(2) Identification of the test rule under which the dispute arose.

(3) A list of the parties from whom reimbursement is sought or who are seeking reimbursement, a brief description of the attempts to reach agreement and a concise explanation of the issues on which the parties are unable to agree.

(c) The request for a hearing shall be accompanied by the appropriate administrative fee, as provided in a current Fee Schedule of the American Arbitration Association.

### § 791.22 Consolidation of hearings.

(a) Promptly upon receipt of the request for a hearing, the Administrator will publish a notice in the FEDERAL REGISTER, advising those subject to the test rule that a request for a hearing has been made.

(b) Any other person wishing to participate in the hearing shall so notify EPA within 45 days of the FEDERAL REGISTER notice. EPA will promptly inform the regional office of the American Arbitration Association where the request has been filed of the additional parties.

### § 791.27 Pre-hearing preparation.

(a) *Responses to requests for hearings.* After filing of the request for hearing, if any other party desires to file an answer it shall be made in writing and filed with the American Arbitration Association, and a copy thereof shall be mailed to the other parties within a period of fourteen days from the date of receiving the complete list of parties. After the hearing officer is appointed, however, no new or different claim may be submitted except with the hearing officer's consent.

(b) *Pre-hearing conference.* At the request of the parties or at the discretion of the American Arbitration Association, a pre-hearing conference with a representative of the American Arbitration Association and the parties or their counsel will be scheduled in appropriate cases to arrange for an exchange of information and the stipulation of uncontested facts so as to expedite the proceedings.

(c) *Fixing of locale.* The parties may mutually agree on the locale where the hearing is to be held. If the locale is not designated within 45 days from the time the complete list of parties is received, the American Arbitration Association shall have power to determine the locale. Its decision shall be final and binding. If any party requests, and informs the other parties of its request, that the hearing be held in a specific locale and the other parties file no objection thereto within 14 days of the request, the locale shall be the one requested.

(d) *Time and place.* The hearing officer shall fix the time and place for each hearing. The American Arbitration Association will mail notice to each party at least 14 days in advance.

### § 791.29 Appointment of hearing officer.

(a) *Qualifications of hearing officer.* All hearing officers shall be neutral, subject to disqualification for the reasons specified in paragraph (f) of this section.

(b) *Appointment from panel.* Promptly after receiving the complete list of parties at the close of the notice period described in § 791.22, the American Arbitration Association shall submit simultaneously to each party to the dispute an identical list of names. Each party to the dispute shall have thirty days from the mailing date in which to cross off any names objected to, number the remaining names to indicate the order of preference, and return the list to the American Arbitration Association. If a party does not return the list within the time specified, all persons named therein shall be deemed acceptable to that party. From among the persons who have been approved on all lists, and in accordance with the designated order of mutual preference, the American Arbitration Association shall invite the acceptance of a hearing officer to serve. If the parties fail to agree upon any of the persons named, or if acceptable hearing officers are unable to act, or if for any other reason the appointment cannot be made from the submitted lists, the American Arbitration Association shall have the power to make the appointment without the submission of any additional list.

(c) *Nationality of hearing officer in international dispute.* If one of the parties is a national or resident of a country other than the United States, the hearing officer shall upon the request of any party, be appointed from among the nationals of a country other than that of the parties.

(d) *Number of hearing officers.* The dispute shall be heard and determined by one hearing officer unless the American Arbitration Association, in its discretion, directs that a greater number of hearing officers be appointed.

(e) *Notice of appointment.* Notice of the appointment of the hearing officer, together with a copy of these rules, and the signed acceptance of the hearing officer shall be filed prior to the opening of the first hearing.

(f) *Disclosure and challenge procedure.* A person appointed as hearing officer shall disclose to the American Arbitration Association any circumstances likely to affect impartiality, including any bias or any financial or personal interest in the result of the hearing or any past or present relationship with the parties or their counsel. Upon receipt of such information from such hearing officer or other source, the American Arbitration Association shall communicate such information to the parties, and, if it deems it appropriate to do so, to the hearing officer and others. Thereafter, the American Arbitration Association shall determine whether the hearing officer should be disqualified and shall inform the parties of its decision, which shall be conclusive.

(g) *Vacancies.* If any hearing officer should resign, die, withdraw, refuse, be disqualified or be unable to perform the duties of the office, the American Arbitration Association may, on proof satisfactory to it, declare the office vacant. Vacancies shall be filled in accordance with the applicable provisions of these rules and the matter shall be reheard unless the parties shall agree otherwise.

### § 791.30 Hearing procedures.

(a) *Representation by counsel.* Any party may be represented by counsel. A party intending to be so represented shall notify the other parties and the American Arbitration Association of the name and address of counsel at least 5 days prior to the date set for the hearing at which counsel is first to appear. When a hearing is initiated by counsel, or where an attorney replies for the other party, such notice is deemed to have been given.

(b) *Stenographic record.* The American Arbitration Association shall make the necessary arrangements for the taking of a stenographic record. The parties shall share the cost of such record.

(c) *Attendance at hearings.* The hearing officer shall have the power to require the exclusion of anyone, including a party or other essential person, during the testimony of any witness to protect confidential business information. It shall be discretionary with the hearing officer to determine the propriety of the attendance of any other person.

(d) *Oaths.* Hearing officers shall swear or affirm their neutrality and their dedication to a fair and equitable resolution. Witnesses shall swear or affirm that they are telling the truth.

(e) *Order of proceedings.* (1) A hearing shall be opened by the filing of the oath of the hearing officer and by the recording of the place, time and date of the hearing, the presence of the hearing officer and parties, and counsel, if any, and by the receipt by the hearing officer of the request for hearing and answer, if any.

(2) The hearing officer may, at the beginning of the hearing, ask for statements clarifying the issues involved.

(3) The party or parties seeking reimbursement shall then present a claim and proofs and witnesses, who shall submit to questions or other examination. The party or parties from whom reimbursement is sought shall then present a defense and proofs and witnesses, who shall submit to questions or other examination. The hearing officer has discretion to vary this procedure but shall afford full and equal opportunity to all parties for the presentation of any material or relevant proofs.

(4) Exhibits, when offered by any party, shall be received in evidence by the hearing officer. The names and addresses of all witnesses and exhibits in order received shall be made a part of the record.

(f) *Hearing in the absence of a party.* A hearing may proceed in the absence of any party which, after due notice, fails to be present or fails to obtain an adjournment. An award shall not be made solely on the default of a party. The hearing officer shall require the parties who are present to submit such evidence as the hearing officer may require for the making of an award.

(g) *Evidence.* (1) The parties may offer such evidence as they desire and shall produce such additional evidence as the hearing officer may deem necessary to an understanding and determination of the dispute. The hearing officer shall be the judge of the relevancy and materiality of the evidence offered and conformity to legal rules of evidence shall not be necessary. All evidence shall be taken in the presence of all the hearing officers and of all the parties, except where any of the parties is absent in default, has waived the right to be present, or has been excluded by the hearing officer to protect confidential business information.

(2) All documents not filed with the hearing officer at the hearing, but arranged for by agreement of the par-

ties, shall be filed with the American Arbitration Association for transmission to the hearing officer, according to the agreed-upon schedule. All parties shall be afforded opportunity to examine such documents.

(h) *Evidence by affidavit and filing of documents.* The hearing officer shall receive and consider the evidence of witnesses by affidavit, but shall give it only such weight as the hearing officer deems it entitled to after consideration of any objections made to its admission.

(i) *Closing of hearings.* The hearing officer shall specifically inquire of all parties whether they have any further proofs to offer or witnesses to be heard. Upon receiving negative replies, the hearing officer shall declare the hearings closed and record the time of closing of the hearing. If briefs are to be filed, the hearings shall be declared closed as of the final date set by the hearing officer for the receipt of briefs. If documents are to be filed as provided for in paragraph (g)(2) of this section and the date set for their receipt is later than that set for the receipt of briefs, the later date shall be the date of closing the hearings.

(j) *Reopening of hearings.* The hearings may be reopened on the hearing officer's own motion, or upon application of a party at any time before the award is made. If the reopening of the hearings would prevent the making of the award within the specified time the matter may not be reopened, unless the parties agree upon the extension of the time limit.

(k) *Waiver of oral hearings.* The parties may provide, by written agreement, for the waiver of oral hearings. If the parties are unable to agree as to the procedure, the American Arbitration Association shall specify a fair and equitable procedure.

(l) *Waiver of rules.* Any party who proceeds with the hearing after knowledge that any provision or requirement of these rules has not been complied with and who fails to state objection thereto in writing, shall be deemed to have waived the right to object.

(m) *Extensions of time.* The parties may modify any period of time by mutual agreement. The American Arbitration Association for good cause may extend any period of time established by these rules, except the time for making the award. (§ 791.37(a)) The American Arbitration Association shall notify the parties of any such extension of time and its reason therefor.

(n) *Communication with hearing officer.* There shall be no direct communication between the parties and a hearing officer other than at oral hearings. Any other oral or written communications from the parties to the hearing officer shall be directed to the American Arbitration Association for transmittal to the hearing officer.

### § 791.31 Expedited procedures.

Unless the American Arbitration Association in its discretion determines otherwise, the Expedited Procedures described in this section shall be applied in any case where the total claim of any party does not exceed $5,000, exclusive of interest and hearing costs, and may be applied in other cases if the parties agree.

(a) *Application of rules.* The expedited hearings will be conducted according to the same procedures as the regular ones, except for those specifically changed by the expedited rules in this section, § 791.31.

(b) *Notice by telephone.* The parties shall accept all notices from the American Arbitration Association by telephone. Such notices by the American Arbitration Association shall subsequently be confirmed in writing to the parties. Notwithstanding the failure to confirm in writing any notice or objection hereunder, the proceeding shall nonetheless be valid if notice or obligation has, in fact, been given by telephone.

(c) *Appointment and qualifications of hearing officers.* The American Arbitration Association shall submit simultaneously to each party to the dispute an identical list of five persons from which one hearing officer shall be appointed. Each party shall have the right to strike two names from the list on a peremptory basis. The list is returnable to the American Arbitration Association within 10 days from the date of mailing. If for any reasons the appointment cannot be made from the list, the American Arbitration Association shall have the authority to make the appointment without the submission of additional lists. Such appointment shall be subject to disqualification for the reasons specified in § 791.29(f). The parties shall be given notice by telephone by the American Arbitration Association of the appointment of the hearing officer. The parties shall notify the American Arbitration Association, by telephone, within 7 days of any objections to the hearing officer(s) appointed. Any objection by a party to such hearing officer shall be confirmed in writing to the American Arbitration Association with a copy to the other parties.

(d) *Time and place of hearing.* The hearing officer shall fix the date, time and place of the hearing. The American Arbitration Association will notify the parties by telephone, 7 days in advance of the hearing date. Formal notice of hearing will be sent by the American Arbitration Association to the parties.

(e) *The hearing.* Generally, the hearing shall be completed within 1 day. The hearing officer, for good cause shown, may schedule an additional hearing to be held within 5 days.

(f) *Time of award.* Unless otherwise agreed to by the parties, the Award shall be rendered not later than 15 business days from the date of the closing of the hearing.

### § 791.34 Serving of notice.

(a) Each party shall be deemed to have consented that any papers, notices or process necessary or proper for the initiation or continuation of a hearing under these rules and for any appeal to EPA or any court action in connection therewith may be served upon such party by mail addressed to such party or its attorney at its last known address or by personal service, within or without the state wherein the arbitration is to be held (whether such party be within or without the United States of America), provided that reasonable opportunity to be heard with regard thereto has been granted such party.

(b) The American Arbitration Association shall, upon the written request of a party, furnish to such party, at its expense, certified facsimiles of any papers in the American Arbitration Association's possession that may be required in appeal to EPA or judicial proceedings relating to the hearing.

### § 791.37 The award.

(a) *Time of award.* The award shall be made promptly by the hearing officer and, unless otherwise agreed by the parties, no later than 30 days from the date of closing the hearings, or if oral hearings have been waived, from the date of transmitting the final statements and proofs to the hearing officer.

(b) *Form of award.* The award shall be in writing and shall be signed either by the sole hearing officer or by at least a majority if there is more than one. It shall contain a concise statement of its basis and rationale, and a timetable for payment of any ordered reimbursement.

(c) *Delivery of award to parties.* Parties shall accept as legal delivery of the award the delivery of the award or a true copy thereof by certified mail to the party at its last known address or to its attorney, or by personal service.

### § 791.39 Fees and expenses.

(a) *Administrative fees.* (1) As a not-for-profit organization, the American Arbitration Association shall prescribe an Administrative Fee Schedule and a Refund Schedule to compensate it for the cost of providing administrative services. The schedule in effect at the time of filing or the time of refund shall be applicable.

(2) The administrative fees shall be advanced by the initiating party or parties, subject to final apportionment by the hearing officer in the award. The administrative fee is increased by 10 percent of the original for each additional party.

(3) Fees and expenses in excess of the limit contained in section 26(b) of TSCA ($2,500 per person, or $100 per small business) will be paid by EPA.

(b) *Expenses.* Subject to paragraph (a)(3) of this section, all expenses of the hearing, including the cost of recording (though not transcribing) the hearing and required traveling and other expenses of the hearing officer and of American Arbitration Association representatives, and the expenses of any witness or the cost of any proofs produced at the direct request of the hearing officer, shall be borne equally by the parties, unless they agree otherwise, or unless the hearing officer, in the award, assesses such expenses or any part thereof against any specified party or parties.

(c) *Hearing officer's fee.* Hearing officers will normally serve without a fee. In prolonged or special cases the American Arbitration Association in consultation with the Administrator may determine that payment of a fee by the parties is appropriate and may establish a reasonable amount, taking into account the extent of service by the hearing officer and other relevant circumstances of the case. Any arrangements for compensation shall be made through the American Arbitration Association and not directly between the parties and the hearing officer.

## Subpart C—Basis for Proposed Order

### § 791.40 Basis for the proposed order.

(a) The hearing officer shall propose a fair and equitable amount of reimbursement. The formula in paragraph (b) of this section shall be presumed to be fair and equitable as applied to all persons subject to a test rule. However, the hearing officer has the discretion to modify the formula, or to use some other basis for allocation if necessary. Additional factors that may be taken into account include, but are not limited to, relative amounts of exposure attributable to each person and the effect of the reimbursement share on competitive position.

(b) In general, each person's share of the test cost shall be in proportion to its share of the total production volume of the test chemical:

$$R_x = C\frac{V_x}{V_t}$$

Where:

R=the reimbursement share owed by company X.

C=the total cost of the testing required by the test rule.

$V_x$=the volume of the test chemical produced or imported by company X over the period defined by § 791.48.

$V_t$=the total volume of the test chemical produced or imported over the period defined by § 791.48.

(c) The burden of proposing modifications to the formula shall lie with the party requesting the modification.

### § 791.45 Processors.

(a) Generally, processors will be deemed to have fulfilled their testing and reimbursement responsibilities indirectly, through higher prices passed on by those directly responsible, the manufacturers. There are three circumstances in which processors will have a responsibility to provide reimbursement directly to those paying for the testing:

(1) When a test rule or subsequent FEDERAL REGISTER notice pertaining to a test rule expressly obligates processors as well as manufacturers to assume direct testing and data reimbursement responsibilities.

(2) When one or more manufacturers demonstrate to the hearing officer that it is necessary to include processors in order to provide fair and equitable reimbursement in a specific case.

(3) When one or more processors voluntarily agree to reimburse manufacturers for a portion of test costs. Only those processors who volunteer will incur the obligation.

(b) A hearing including processors shall be initiated in the same way as those including only manufacturers. Voluntary negotiations must be attempted in good faith first, and the request for a hearing must contain the names of the parties and a description of the unsuccessful negotiations.

(c) When processors as well as manufacturers are required to provide reimbursement, the hearing officer will decide for each case how the reimbursement should be allocated among the participating parties. When a test rule is applicable solely to processors, the hearing officer will apply the formula to the amount of the test chemical purchased or processed.

### § 791.48 Production volume.

(a) Production volume will be measured over a period that begins one calendar year before publication of the final test rule in the FEDERAL REGISTER and continues up to the latest data available upon resolution of a dispute.

(b) For the purpose of determining fair reimbursement shares, production volume shall include amounts of the test chemical imported in bulk form and mixtures, and the total domestic production of the chemical including that produced as a byproduct. Impurities will not be included unless the test rule specifically includes them.

(c) Amounts of the test chemical manufactured for export will not be included unless covered by a finding under TSCA section 12(a)(2).

(d) Chemicals excluded from the jurisdiction of TSCA by section 3(2)(B) need not be included in the computation of production volume. (Chemicals used as intermediates to produce pesticides are covered by TSCA.)

(e) The burden of establishing the fact that particular amounts of the test chemical are produced for exempt purposes lies with the party seeking to exclude those amounts from the calculation of his production volume.

### § 791.50 Costs.

(a) All costs reasonable and necessary to comply with the test rule, taking into account the practices of other laboratories in conducting similar tests, are eligible for reimbursement. Necessary costs include:

(1) Direct and indirect costs of planning, conducting, analyzing and submitting the test results to EPA.

(2) A reasonable profit, and a reasonable rate of interest and depreciation on the tester's initial capital investment.

(3) The cost of repeating or repairing tests where failure was demonstrably due to some cause other than negligence of the tester.

(b) Costs attributable to tests beyond those specified by EPA shall not be eligible for reimbursement under this rule.

### § 791.52 Multiple tests.

When more than one of a particular kind of test required by the test rule is performed, the additional costs will be shared among all those holding exemptions. The costs of all the tests will be added together and each exemption holder shall be responsible for a share of the total which is equal to its share of the total production of the test chemical. The exemption holders shall divide their shares between test sponsors in proportion to the costs of their respective tests. Those sponsoring a particular test do not have to obtain exemptions for that test and therefore do not have reimbursement responsibilities for the same tests done by others.

## Subpart D—Review

### § 791.60 Review.

(a) The hearing officer's proposed order shall become the final Agency order 30 days after issuance unless within the 30-day period one of the parties requests Agency review or the Administrator of his own initiative decides to review the proposed order.

(b) The proposed order may be reviewed upon the record of the hearing and the petitions for review. If necesary, the Administrator may order the transcription of the stenographic record of the hearing, written briefs, oral arguments or any other reasonable aids to making an equitable decision.

(c) The final Agency order may be reviewed in federal court as provided by 26 U.S.C. 2603(c).

## Subpart E—Final Order

### § 791.85 Availablity of final Agency order.

The final Agency order shall be available to the public for inspection and copying pursuant to 5 U.S.C. 552(a)(2), subject to necessary confidentiality restrictions.

## Subpart F—Prohibited Acts

### § 791.105 Prohibited acts.

Failure to provide information required by the Agency or to pay the amounts awarded under this rule within time alloted in the final order shall constitute a violation of 15 U.S.C. 2614(1) or 2614(3).

# PART 792—GOOD LABORATORY PRACTICE STANDARDS

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AUTHORITY: 15 U.S.C. 2603.

SOURCE: 48 FR 53937, Nov. 29, 1983, unless otherwise noted.

## Subpart A—General Provisions

### § 792.1 Scope.

(a) This part prescribes good laboratory practices for conducting studies relating to health effects, environmental effects, and chemical fate testing. This part is intended to assure the quality and integrity of data submitted pursuant to section 4(a) of the Toxic Substances Control Act (TSCA) (Pub. L. 94-469, 90 Stat. 2006, 15 U.S.C., 2603 *et seq.*).

(b) This part applies to any study described by paragraph (a) of this section which any person conducts, initiates, or supports on or after December 29, 1983.

(c) It is the Agency's policy that all data developed under section 5 of TSCA should be in accordance with the provisions of this part. It is also EPA policy to incorporate this part into all chemical-specific negotiated testing agreements. If data are not developed in accordance with the provisions of this part, the Agency will consider such data insufficient to evaluate the health and environmental effects of the chemical substances unless the submitter provides additional information demonstrating that the data are reliable and adequate.

**§ 792.3 Definitions.**

As used in this part the following terms shall have the meanings specified:

(a) [Reserved]

(b) "Batch" means a specific quantity or lot of a test or control substance that has been characterized according to § 792.105(a).

(c) "Control substance" means any chemical substance or mixture or any other material other than a test substance that is administered to the test system in the course of a study for the purpose of establishing a basis for comparison with the test substance.

(d) "EPA" means the U.S. Environmental Protection Agency.

(e) "FDA" means the U.S. Food and Drug Administration.

(f)-(g) [Reserved]

(h) "Person" includes an individual, partnership, corporation, association, scientific or academic establishment, government agency, or organizational unit thereof, and any other legal entity.

(i) "Quality assurance unit" means any person or organizational element, except the study director, designated by testing facility management to perform the duties relating to quality assurance of the studies.

(j) "Raw data" means any laboratory worksheets, records, memoranda, notes, or exact copies thereof, that are the result of original observations and activities of a study and are necessary for the reconstruction and evaluation of the report of that study. In the event that exact transcripts of raw data have been prepared (e.g., tapes which have been transcribed verbatim, dated, and verified accurate by signature), the exact copy or exact transcript may be substituted for the original source as raw data. "Raw data" may include photographs, microfilm or mocrofiche copies, computer printouts, magnetic media, including dictated observations, and recorded data from automated instruments.

(k) "Specimen" means any material derived from a test system for examination or analysis.

(l) "Sponsor" means:

(1) A person who initiates and supports, by provision of financial or other resources, a study;

(2) A person who submits a study to the EPA in response to a section 4(a) test rule and/or a person who submits a study under a negotiated testing agreement or section 5 rule/order to the extent the agreement or rule/order references this part; or

(3) A testing facility, if it both initiates and actually conducts the study.

(m) "Study" means any *in vivo* or *in vitro* experiment in which a test substance is studied prospectively in a test system under conditions to determine or help predict its fate, toxicity, metabolism, or other characteristics in humans, other animals and plants. The term does not include studies utilizing human subjects or clinical studies. The term does not include basic exploratory studies carried out to determine whether a test substance has any potential utility.

(n) "Study director" means the individual responsible for the overall conduct of a study.

(o) "Test substance or mixture" means a substance or mixture administered or added to a test system in a study, which substance or mixture is used to develop data to meet the requirements of a TSCA section 4(a) test rule and/or is developed under a negotiated testing agreement or section 5 rule/order to the extent the agreement or rule/order references this part.

(p) "Test system" means any animal, plant, microorganism, or subparts thereof, to which the test or control substance is administered or added for study. "Test system" also includes appropriate groups or components of the system not treated with the test or control substances.

(q) "Testing facility" means a person who actually conducts a study, i.e., actually uses the test substance in a test system. "Testing facility" encompasses only those operational units that are being or have been used to conduct studies.

(r) "TSCA" means the Toxic Substances Control Act (15 U.S.C. 2601 *et seq.*)

### § 792.10 Applicability to studies performed under grants and contracts.

When a sponsor or other person utilizes the services of a consulting laboratory, contractor, or grantee to perform all or a part of a study to which this part applies, it shall notify the consulting laboratory, contractor, or grantee that the service is, or is part of, a study that must be conducted in compliance with the provisions of this part.

### § 792.12 Statement of compliance or noncompliance.

Any person who submits to EPA a test required by a rule promulgated under section 4 of TSCA shall include in the submission a true and correct statement, signed by the sponsor and the study director, of one of the following types:

(a) A statement that the study was conducted in accordance with this part; or

(b) A statement describing in detail all differences between the practices used in the study and those required by this part; or

(c) A statement that the person was not a sponsor of the study, did not conduct the study, and does not know whether the study was conducted in accordance with this part.

### § 792.15 Inspection of a testing facility.

(a) A testing facility shall permit an authorized employee or duly designated representative of EPA or FDA, at reasonable times and in a reasonable manner, to inspect the facility and to inspect (and in the case of records also to copy) all records and specimens required to be maintained regarding studies to which this part applies. The records inspection and copying requirements shall not apply to quality assurance unit records of findings and problems, or to actions recommended and taken, except that EPA may seek production of these records in litigation or formal adjudicatory hearings.

(b) EPA will not consider reliable for purposes of showing that a chemical substance or mixture does not present a risk of injury to health or the environment any data developed by a testing facility or sponsor that refuses to permit inspection in accordance with this part. The determination that a study will not be considered reliable does not, however, relieve the sponsor of a required test of any obligation under any applicable statute or regulation to submit the results of the study to EPA.

(c) Since a testing facility is a place where chemicals are stored or held, it is subject to inspection under section 11 of TSCA.

### § 792.17 Effects of non-compliance.

(a) The sponsor or any other person who is conducting or has conducted a test to fulfill the requirements of a test rule promulgated under section 4 of TSCA will be in violation of section 15 of TSCA if:

(1) The test is not being or was not conducted in accordance with any requirement of this part;

(2) Data or information submitted to EPA under this part (including the statement required by § 792.12) include information or data that are false or misleading, contain significant omissions, or otherwise do not fulfill the requirements of this part; or

(3) Entry in accordance with § 792.15 for the purpose of auditing test data or inspecting test facilities is denied. Persons who violate the provisions of this part may be subject to civil or criminal penalties under section 16 of TSCA, legal action in United States district court under section 17 of TSCA, or criminal prosecution under 18 U.S.C. 2 or 1001.

(b) EPA, at its discretion, may not consider reliable for purposes of showing that a chemical substance or mixture does not present a risk of injury to health or the environment any study which was not conducted in accordance with this part. EPA, at its discretion, may rely upon such studies for purposes of showing adverse effects. The determination that a study will not be considered reliable does not, however, relieve the sponsor of a required test of the obligation under any applicable statute or regulation to submit the results of the study to EPA.

(c) If data submitted to fulfill a requirement of a test rule under section 4 of TSCA is not developed in accordance with this part, EPA may determine that the sponsor has not fulfilled its obligations under section 4 of TSCA and may require the sponsor to develop data in accordance with the requirements of this part in order to satisfy such obligations.

## Subpart B—Organization and Personnel

### § 792.29 Personnel.

(a) Each individual engaged in the conduct of or responsible for the supervision of a study shall have education, training, and experience, or combination thereof, to enable that individual to perform the assigned functions.

(b) Each testing facility shall maintain a current summary of training and experience and job description for each individual engaged in or supervising the conduct of a study.

(c) There shall be a sufficient number of personnel for the timely and proper conduct of the study according to the protocol.

(d) Personnel shall take necessary personal sanitation and health precautions designed to avoid contamination of test and control substances and test systems.

(e) Personnel engaged in a study shall wear clothing appropriate for the duties they perform. Such clothing shall be changed as often as necessary to prevent microbiological, radiological, or chemical contamination of test systems and test and control substances.

(f) Any individual found at any time to have an illness that may adversely affect the quality and integrity of the study shall be excluded from direct contact with test systems, test and control substances and any other operation or function that may adversely affect the study until the condition is corrected. All personnel shall be instructed to report to their immediate supervisors any health or medical conditions that may reasonably be considered to have an adverse effect on a study.

### § 792.31 Testing facility management.

For each study, testing facility management shall:

(a) Designate a study director as described in § 792.33 before the study is initiated.

(b) Replace the study director promptly if it becomes necessary to do so during the conduct of a study, and document and maintain such action as raw data.

(c) Assure that there is a quality assurance unit as described in § 792.35.

(d) Assure that test and control substances or mixtures have been appropriately tested for identity, strength, purity, stability, and uniformity, as applicable.

(e) Assure that personnel, resources, facilities, equipment, materials and methodologies are available as scheduled.

(f) Assure that personnel clearly understand the functions they are to perform.

(g) Assure that any deviations from these regulations reported by the quality assurance unit are communicated to the study director and corrective actions are taken and documented.

### § 792.33 Study director.

For each study, a scientist or other professional of appropriate education, training, and experience, or combination thereof, shall be identified as the study director. The study director has overall responsibility for the technical conduct of the study, as well as for the interpretation, analysis, documentation, and reporting of results, and represents the single point of study control. The study director shall assure that:

(a) The protocol, including any change, is approved as provided by § 792.120 and is followed.

(b) All experimental data, including observations of unanticipated responses of the test system are accurately recorded and verified.

(c) Unforeseen circumstances that may affect the quality and integrity of the study are noted when they occur, and corrective action is taken and documented.

(d) Test systems are as specified in the protocol.

(e) All applicable good laboratory practice regulations are followed.

(f) All raw data, documentation, protocols, specimens, and final reports are transferred to the archives during or at the close of the study.

### § 792.35 Quality assurance unit.

(a) A testing facility shall have a quality assurance unit composed of one or more individuals who shall be responsible for monitoring each study to assure management that the facilities, equipment, personnel, methods, practices, records, and controls are in conformance with the regulations in this part. For any given study the quality assurance unit shall be entirely separate from and independent of the personnel engaged in the direction and conduct of that study.

(b) The quality assurance unit shall:

(1) Maintain a copy of a master schedule sheet of all studies conducted at the testing facility indexed by test substance and containing the test system, nature of study, date study was initiated, current status of each study, name of the sponsor, name of the study director, and status of the final report.

(2) Maintain copies of all protocols pertaining to all studies for which the unit is responsible.

(3) Inspect each phase of a study periodically and maintain written and properly signed records of each periodic inspection showing the date of the inspection, the study inspected, the phase or segment of the study inspected, the person performing the inspection, findings and problems, action recommended and taken to resolve existing problems, and any scheduled date for re-inspection. For studies lasting more than six months, inspections shall be conducted every three months. For studies lasting less than six months, inspections shall be conducted at intervals adequate to assure the integrity of the study. Any significant problems which are likely to affect study integrity found during the course of an inspection shall be brought to the attention of the study director and management immediately.

(4) Periodically submit to management and the study director written status reports on each study, noting any problems and the corrective actions taken.

(5) Determine that no deviations from approved protocols or standard operating procedures were made without proper authorization and documentation.

(6) Review the final study report to assure that such report accurately describes the methods and standard operating procedures, and that the reported results accurately reflect the raw data of the study.

(7) Prepare and sign a statement to be included with the final study report which shall specify the dates inspections were made and findings reported to management and to the study director.

(c) The responsibilities and procedures applicable to the quality assurance unit, the records maintained by the quality assurance unit, and the method of indexing such records shall be in writing and shall be maintained. These items including inspection dates, the study inspected, the phase or segment of the study inspected, and the name of the individual performing the inspection shall be made available for inspection to authorized employees or duly designated representatives of EPA or FDA.

(d) An authorized employee or a duly designated representative of EPA or FDA shall have access to the written procedures established for the inspection and may request testing facility management to certify that inspections are being implemented, performed, documented and followed-up in accordance with this paragraph.

(e) All records maintained by the quality assurance unit shall be kept in one location at the testing facility.

## Subpart C—Facilities

### § 792.41 General.

Each testing facility shall be of suitable size, construction, and location to facilitate the proper conduct of studies. It shall be designed so that there is a degree of separation that will prevent any function or activity from having an adverse effect on the study.

**§ 792.43 Animal care facilities.**

(a) A testing facility shall have a sufficient number of animal rooms or areas, as needed, to assure proper: (1) Separation of species or test systems, (2) isolation of individual projects, (3) quarantine of animals, and (4) routine or specialized housing of animals.

(b) A testing facility shall have a number of animal rooms or areas separate from those described in paragraph (a) of this section to ensure isolation of studies being done with test systems or test and control substances known to be biohazardous, including volatile substances, aerosols, radioactive materials, and infectious agents.

(c) Separate areas shall be provided for the diagnosis, treatment, and control of laboratory animal diseases. These areas shall provide effective isolation for the housing of animals either known or suspected of being diseased, or of being carriers of disease, from other animals.

(d) When animals are housed, facilities shall exist for the collection and disposal of all animal waste and refuse or for safe sanitary storage of waste before removal from the testing facility. Disposal facilities shall be so provided and operated as to minimize vermin infestation, odors, disease hazards, and environmental contamination.

(e) Animal facilities shall be designed, constructed, and located so as to minimize disturbances that interfere with the study.

**§ 792.45 Animal supply facilities.**

There shall be storage areas, as needed, for feed, bedding, supplies, and equipment. Storage areas for feed and bedding shall be separated from areas housing the test systems and shall be protected against infestation or contamination. Refrigeration shall be provided for perishable supplies or feed.

**§ 792.47 Facilities for handling test and control substances.**

(a) As necessary to prevent contamination or mixups, there shall be separate areas for:

(1) Receipt and storage of the test and control substances.

(2) Mixing of the test and control substances with a carrier, e.g., feed.

(3) Storage of the test and control substance mixtures.

(b) Storage areas for the test and/or control substance and test and control mixtures shall be separate from areas housing the test systems and shall be adequate to preserve the identity, strength, purity, and stability of the substances and mixtures.

**§ 792.49 Laboratory operation areas.**

(a) Separate laboratory space shall be provided, as needed, for the performance of the routine procedures required by studies, including specialized areas for performing activities such as aseptic surgery, intensive care, necropsy, histology, radiography, and handling of biohazardous materials.

(b) Separate space shall be provided for cleaning, sterilizing, and maintaining equipment and supplies used during the course of the study.

**§ 792.51 Specimen and data storage facilities.**

Space shall be provided for archives, limited to access by authorized personnel only, for the storage and retrieval of all raw data and specimens from completed studies.

**§ 792.53 Administrative and personnel facilities.**

(a) There shall be space provided for the administration, supervision, and direction of the testing facility.

(b) Separate space shall be provided for locker, shower, toilet, and washing facilities, as needed.

## Subpart D—Equipment

**§ 792.61 Equipment design.**

Automatic, mechanical, or electronic equipment used in the generation, measurement, or assessment of data and equipment used for facility environmental control shall be of appropriate design and adequate capacity to function according to the protocol and shall be suitably located for operation, inspection, cleaning, and maintenance.

### § 792.63 Maintenance and calibration of equipment.

(a) Equipment shall be adequately inspected, cleaned, and maintained. Equipment used for the generation, measurement, or assessment of data shall be adequately tested, calibrated, and/or standardized.

(b) The written standard operating procedures required under § 792.81(b)(11) shall set forth in sufficient detail the methods, materials, and schedules to be used in the routine inspection, cleaning, maintenance, testing, calibration, and/or standardization of equipment, and shall specify remedial action to be taken in the event of failure or malfunction of equipment. The written standard operating procedures shall designate the person responsible for the performance of each operation, and copies of the standard operating procedures shall be made available to laboratory personnel.

(c) Written records shall be maintained of all inspection, maintenance, testing, calibrating, and/or standardizing operations. These records, containing the date of the operation, shall describe whether the maintenance operations were routine and followed the written standard operating procedures. Written records shall be kept of nonroutine repairs performed on equipment as a result of failure and malfunction. Such records shall document the nature of the defect, how and when the defect was discovered, and any remedial action taken in response to the defect.

## Subpart E—Testing Facilities Operation

### § 792.81 Standard operating procedures.

(a) A testing facility shall have standard operating procedures in writing, setting forth study methods that management is satisfied are adquate to insure the quality and integrity of the data generated in the course of a study. All deviations in a study from standard operating procedures shall be authorized by the study director and shall be documented in the raw data. Significant changes in established standard operating procedures shall be properly authorized in writing by management.

(b) Standard operating procedures shall be established for, but not limited to, the following:

(1) Animal room preparation.

(2) Animal care.

(3) Receipt, indentification, storage, handling, mixing, and method of sampling of the test and control substances.

(4) Test system observations.

(5) Laboratory test.

(6) Handling of animals found moribund or dead during study.

(7) Necropsy of animals or postmortem examination of animals.

(8) Collection and indentification of specimens.

(9) Histopathology.

(10) Data handling, storage, and retrieval.

(11) Maintenance and calibration of equipment.

(12) Transfer, proper placement, and identification of animals.

(c) Each laboratory area shall have immediately available laboratory manuals and standard operating procedures relative to the laboratory procedures being performed, e.g., toxicology, histology, clinical chemistry, hematology, teratology, necropsy. Published literature may be used as a supplement to standard operating procedures.

(d) A historical file of standard operating procedures, and all revisions thereof, including the dates of such revisions, shall be maintained.

### § 792.83 Reagents and solutions.

All reagents and solutions in the laboratory areas shall be labeled to indicate identity, titer or concentration, storage requirements, and expiration date. Deteriorated or outdated reagents and solutions shall not used.

### § 792.90 Animal care.

(a) There shall be standard operating procedures for the housing, feeding, handling, and care of animals.

(b) All newly received animals from outside sources shall be placed in quarantine until their health status has been evaluated. This evaluation shall be in accordance with acceptable veterinary medical practice.

(c) At the initiation of a study, animals shall be free of any disease or condition that might interfere with the purpose or conduct of the study. If, during the course of the study, the animals contract such a disease or condition, the diseased animals shall be isolated. If necessary, these animals may be treated for disease or signs of disease provided that such treatment does not interfere with the study. The diagnosis, authorization of treatment, description of treatment and each date of treatment shall be documented and shall be retained.

(d) Warm-blooded animals, excluding suckling rodents, used in laboratory procedures that require manipulations and observations over an extended period of time or in studies that require the animals to be removed from and returned to their home cages for any reason (e.g., cage cleaning, treatment, etc.), shall receive appropriate identification (e.g., tattoo, toe clip, color code, ear tag, ear punch, etc.). All information needed to specifically identify each animal within an animal-housing unit shall appear on the outside of that unit.

(e) Animals of different species shall be housed in separate rooms when necessary. Animals of the same species, but used in different studies, should not ordinarily be housed in the same room when inadvertent exposure to control or test substances or animal mixup could affect the outcome of either study. If such mixed housing is necessary, adequate differentiation by space and identification shall be made.

(f) Animal cages, racks and accessory equipment shall be cleaned and sanitized at appropriate intervals.

(g) Feed and water used for the animals shall be analyzed periodically to ensure that contaminants known to be capable of interfering with the study and reasonably expected to be present in such feed or water are not present at levels above those specified in the protocol. Documentation of such analyses shall be maintained as raw data.

(h) Bedding used in animal cages or pens shall not interfere with the purpose or conduct of the study and shall be changed as often as necessary to keep the animals dry and clean.

(i) If any pest control materials are used, the use shall be documented. Cleaning and pest control materials that interfere with the study shall not be used.

## Subpart F—Test and Control Substances

### § 792.105 Test and control substance characterization.

(a) The indentity, strength, purity, and composition or other characteristics which will appropriately define the test or control substance shall be determined for each batch and shall be documented before the initiation of the study. Methods of synthesis, fabrication, or derivation of the test and control substances shall be documented by the sponsor or the testing facility.

(b) The stability of each test or control substance shall be determined by the testing facility or by the sponsor before initiation of a study. If the stability of the test or control substances cannot be determined before initiation of a study, standard operating procedures shall be established and followed to provide for periodic reanalysis of each batch.

(c) Each storage container for a test or control substance shall be labeled by name, chemical abstract number (CAS) or code number, batch number, expiration date, if any, and, where appropriate, storage conditions necessary to maintain the identity, strength, purity, and composition of the test or control substance. Storage containers shall be assigned to a particular test substance for the duration of the study.

(d) For studies of more than 4 weeks' duration, reserve samples from each batch of test and control substances shall be retained for the period of time provided by § 792.195.

(e) The stability of test and contol substances under the test conditions shall be known for all studies.

**§ 792.107 Test and control substance handling.**

Procedures shall be established for a system for the handling of the test and control substances to ensure that:

(a) There is proper storage.

(b) Distribution is made in a manner designed to preclude the possibility of contamination, deterioration, or damage.

(c) Proper identification is maintained throughout the distribution process.

(d) The receipt and distribution of each batch is documented. Such documentation shall include the date and quantity of each batch distributed or returned.

**§ 792.113 Mixtures of substances with carriers.**

(a) For each test or control substance that is mixed with a carrier, tests by appropriate analytical methods shall be conducted:

(1) To determine the uniformity of the mixture and to determine, periodically, the concentration of the test or control substance in the mixture.

(2) To determine the stability of the test and control substances in the mixture. If the stability cannot be determined before initiation of the study, standard operating procedures shall be established and followed to provide for periodic re-analysis of the test and control substances in the mixture.

(b) Where any of the components of the test or control substance carrier mixture has an expiration date, that date shall be clearly shown on the container. If more than one component has an expiration date, the earliest date shall be shown.

## Subpart G—Protocol for and Conduct of a Study

**§ 792.120 Protocol.**

(a) Each study shall have an approved written protocol that clearly indicates the objectives and all methods for the conduct of the study. The protocol shall contain but shall not necessarily be limited to the following information:

(1) A descriptive title and statement of the purpose of the study.

(2) Identification of the test and control substance by name, chemical abstract (CAS) number or code number.

(3) The name and address of the sponsor and the name and address of the testing facility at which the study is being conducted.

(4) The proposed starting and completion dates.

(5) Justification for selection of the test system.

(6) Where applicable, the number, body weight range, sex, source of supply, species, strain, substrain, and age of the test system.

(7) The procedure for identification of the test system.

(8) A description of the experimental design, including the methods for the control of bias.

(9) A description and/or identification of the diet used in the study as well as solvents, emulsifiers and/or other materials used to solubilize or suspend the test or control substances before mixing with the carrier. The description shall include specifications for acceptable levels of contaminants that are reasonably expected to be present in the dietary materials and are known to be capable of interfering with the purpose or conduct of the study if present at levels greater than established by the specifications.

(10) The route of administration and the reason for its choice.

(11) Each dosage level, expressed in milligrams per kilogram of body weight or other appropriate units, of the test or control substance to be administered and the method and frequency of administration.

(12) Method by which the degree of absorption of the test and control substances by the test system will be determined if necessary to achieve the objectives of the study.

(13) The type and frequency of test, analyses, and measurements to be made.

(14) The records to be maintained.

(15) The date of approval of the protocol by the sponsor and the signature of the study director.

(16) A statement of the proposed statistical methods to be used.

(b) All changes in or revisions of an approved protocol and the reasons therefor shall be documented, signed by the study director, dated, and maintained with the protocol.

**§ 792.130 Conduct of a study.**

(a) The study shall be conducted in accordance with the protocol.

(b) The test systems shall be monitored in conformity with the protocol.

(c) Specimens shall be identified by test system, study, nature, and date of collection. This information shall be located on the specimen container or shall accompany the specimen in a manner that precludes error in the recording and storage of data.

(d) Records of gross findings for a specimen from postmortem observations shall be available to a pathologist when examining that specimen histopathologically.

(e) All data generated during the conduct of a study, except those that are generated as direct computer input, shall be recorded directly, promptly, and legibly in ink. All data entries shall be dated on the day of entry and signed or initialed by the person entering the data. Any change in entries shall be made so as not to obscure the original entry, shall indicate the reason for such change, and shall be dated and signed or identified at the time of the change. In computer driven data collection systems, the individual responsible for direct data input shall be identified at the time of data input. Any change in computer entries shall be made so as not to obscure the original entry, shall indicate the reason for change, and shall be dated and the responsible individual shall be identified.

## Subparts H and I—[Reserved]

## Subpart J—Records and Reports

**§ 792.185 Reporting of study results.**

(a) A final report shall be prepared for each study and shall include, but not necessarily be limited to, the following:

(1) Name and address of the facility performing the study and the dates on which the study was initiated and was completed, terminated, or discontinued.

(2) Objectives and procedures stated in the approved protocol, including any changes in the original protocol.

(3) Statistical methods employed for analyzing the data.

(4) The test and control substances identified by name, chemical abstracts (CAS) number or code number, strength, purity, and composition or other appropriate characteristics.

(5) Stability of the test and control substances under the conditions of administration.

(6) A description of the methods used.

(7) A description of the test system used. Where applicable, the final report shall include the number of animals or other test organisms used, sex, body weight range, source of supply, species, strain and substrain, age, and procedure used for identification.

(8) A description of the dosage, dosage regimen, route of administration, and duration.

(9) A description of all circumstances that may have affected the quality or integrity of the data.

(10) The name of the study director, the names of other scientists or professionals, and the names of all supervisory personnel, involved in the study.

(11) A description of the transformations, calculations, or operations performed on the data, a summary and analysis of the data, and a statement of the conclusions drawn from the analysis.

(12) The signed and dated reports of each of the individual scientists or other professionals involved in the study, including each person who, at the request or direction of the testing facility or sponsor, conducted an analysis or evaluation of data or specimens from the study after data generation was completed.

(13) The locations where all specimens, raw data, and the final report are to be stored.

(14) The statement prepared and signed by the quality assurance unit as described in § 792.35(b)(7).

(b) The final report shall be signed and dated by the study director.

(c) Corrections or additions to a final report shall be in the form of an amendment by the study director. The amendment shall clearly identify that part of the final report that is being added to or corrected and the reasons for the correction or addition, and shall be signed and dated by the person responsible.

(d) A copy of the final report and of any amendment to it shall be maintained by the sponsor and the testing facility.

### § 792.190 Storage and retrieval of records and data.

(a) All raw data, documentation, records, protocols, specimens, and final reports generated as a result of a study shall be retained. Correspondence and other documents relating to the conduct of a study and the interpretation and evaluation of data, other than those documents contained in the final report, also shall be retained.

(b) There shall be archives for orderly storage and expedient retrieval of all raw data, documentation, protocols, specimens, and interim and final reports. Conditions of storage shall minimize deterioration of the documents or specimens in accordance with the requirements for the time period of their retention and the nature of the documents or specimens. A testing facility may contract with commercial archives to provide a repository for all material to be retained. Raw data and specimens may be retained elsewhere provided that the archives have specific reference to those other locations.

(c) An individual shall be identified as responsible for the archives.

(d) Only authorized personnel shall enter the archives.

(e) Material retained or referred to in the archives shall be indexed by test substance, date of study, test system, and nature of study.

### § 792.195 Retention of records.

(a) Record retention requirements set forth in this section do not supersede the record retention requirements of any other regulations in this subchapter.

(b)(1) Except as provided in paragraph (c) of this section, documentation records, raw data, and specimens pertaining to a study and required to be retained by this part shall be retained in the archive(s) for a period of at least ten years following the effective data of the applicable final test rule.

(2) In the case of negotiated testing agreements, each agreement will contain a provision that, except as provided in paragraph (c) of this section, documentation records, raw data, and specimens pertaining to a study and required to be retained by this part shall be retained in the archive(s) for a period of at least ten years following the publication date of the acceptance of a negotiated test agreement.

(3) In the case of testing submitted under section 5, except for those items listed in paragraph (c) of this section, documentation records, raw data, and specimens pertaining to a study should be retained in the archive(s) for a period of at least five years following the date on which the results of the study are submitted to the Agency.

(c) Wet specimens, samples of test or control substances, and specially prepared material (e.g., histochemical, electron microscopic, blood mounts, teratological preparation, and uteri from dominant lethal mutagenesis tests), which are relatively fragile and differ markedly in stability and quality during storage, shall be retained only as long as the quality of the preparation affords evaluation. In no case shall retention be required for longer periods than those set forth in paragraph (b) of this section.

(d) The master schedule sheet, copies of protocols, and records of quality assurance inspections, as required by § 792.35(c) shall be maintained by the quality assurance unit as an easily accessible system of records for the period of time specified in paragraph (b) of this section.

(e) Summaries of training and experience and job descriptions required to be maintained by § 792.29(b) may be retained along with all other testing facility employment records for the length of time specified in paragraph (b) of this section.

(f) Records and reports of the maintenance and calibration and inspection of equipment, as required by § 792.63 (b) and (c), shall be retained for the length of time specified in paragraph (b) of this section.

(g) If a facility conducting testing or an archive contracting facility goes out of business, all raw data, documentation, and other material specified in this section shall be transferred to the archives of the sponsor of the study. The EPA shall be notified in writing of such a transfer.

(h) Specimens, samples, or other non-documentary materials need not be retained after EPA has notified in writing the sponsor or testing facility holding the materials that retention is no longer required by EPA. Such notification normally will be furnished upon request after EPA or FDA has completed an audit of the particular study to which the materials relate and EPA has concluded that the study was conducted in accordance with this part.

## Subpart K—[Reserved]

## Subpart L—Environmental Testing Provisions

### § 792.225 Applicability of this subpart.

This subpart contains modifications to Subparts A through J that apply to environmental studies.

### § 792.226 Additional definitions applicable to environmental studies.

(a) "Test system" includes, in addition to those systems listed in § 792.3(p), chemical or physical matrices (e.g., soil or water), or subparts thereof, to which the test or control substance or mixture is administered or added for study.

(b) "Carrier" means any material (e.g., feed, water, soil, nutrient media) with which the test substance is combined for administration to test organisms.

(c) "Chemical fate studies" means studies performed for the characterization of physical, chemical, and persistence factors that may be used to evaluate transport and transformation processes.

(d) "Ecological effects studies" means studies performed for the development of information on nonhuman toxicity and potential ecological impact of test substances and their degradation and activation products.

(e) "Environmental studies" refers to either ecological effects studies or chemical fate studies or both.

(f) "Reference substance" means any chemical substance or mixture or material other than a test substance that is administered to or used in analyzing the test system in the course of a study for the purposes of establishing a basis for comparison with the test substance. For purposes of this Subpart all references to "control substance" in Subparts B through J also include "reference substance" as defined herein.

(g) "Vehicle" means any agent which facilitates the mixture, dispersion, or solubilization of a test substance with a carrier.

### § 792.228 Ecological effects testing.

The following additions and modifications to Subparts B through J of this part apply to ecological effects studies.

(a) *Use of the term "animals".* The term "animal(s)" is used in Subparts B through J and shall be construed as including, when appropriate, plant and microbial test organisms.

(b) *Test system facilities.* The following provisions apply to facilities used for ecological effects studies (refer to § 792.43):

(1) In tests with plants or aquatic animals, proper separation of species can be accomplished within a room or area by housing them separately in different chambers or aquaria. Separation of species is unnecessary where the protocol specifies the simultaneous exposure of two or more species in the same chamber, aquarium or housing unit.

(2) Aquatic toxicity tests for individual projects need to be isolated only to the extent necessary to prevent cross-contamination of different chemicals used in different tests.

(3) Facilities shall have proper provisions for collection and disposal of contaminated water, soil or other spent materials.

(4) The facilities shall have provisions to regulate environmental conditions (e.g., temperature, humidity,

photo-period) as specified in the protocol.

(5) For marine test organisms, an adequate supply of clean sea water or artificial sea water (prepared from deionized or distilled water and sea salt mixture) shall be available. The ranges of composition shall be as specified in the protocol.

(6) For freshwater organisms, an adequate, supply of clean water of the appropriate hardness, pH, and temperature, and free of contaminants capable of interfering with the study, shall be available.

(7) For plants, an adequate supply of soil of the appropriate composition, as defined in the protocol, shall be available as needed.

(c) *Test system supply facilities for ecological effects testing* (refer to § 792.45).

(1) When appropriate, plant supply facilities shall be provided. These include:

(i) Storage areas, as needed, for nutrients and soils.

(ii) Facilities for holding, culturing, and maintaining algae and aquatic plants, as specified in the protocol.

(iii) Facilities, as specified in the protocol, for plant growth (e.g., greenhouses, growth chambers, light banks).

(2) Facilities for aquatic animal tests shall be provided. These include aquaria, holding tanks, ponds, and ancillary equipment as specified in the protocol.

(d) *Standard operating procedures.* When appropriate, standard operating procedures shall be established for preparation and maintenance of incubators, greenhouses, or growth chambers.

(e) *Test system care for ecological effects testing.* The following modifications to § 792.90 apply to ecological effects testing:

(1) The health status of test organisms shall be evaluated in accordance with acceptable scientific practice.

(2) Adult reptiles and adult terrestrial amphibians must receive appropriate identification of individuals. Suckling mammals and juvenile birds are excluded from the requirement for individual identification unless otherwise specified in the protocol.

(3) Plants, invertebrate animals, aquatic vertebrate animals, and organisms that may be used in multispecies tests need not be housed in separate rooms, provided that they are adequately segregated to avoid mix-up and cross contamination.

(4) Pens and enclosures, aquaria, holding tanks, ponds, growth chambers, and other holding, rearing and breeding areas are also to be cleaned and sanitized at appropriate intervals.

(5) Soil and water used in plant studies require periodic analysis of contaminants.

(6) All plant and animal test organisms shall be acclimatized, prior to the initiation of the study, to the environmental conditions of the test.

(f) *Mixtures of substances with carriers.* The following modifications apply to the requirements of § 792.113:

(1) Determination of the uniformity of the mixture and of the concentration of the test or control substance in the mixture during the test shall only be accomplished to the extent required by the protocol.

(2) Determination of the stability of test and control substances in the mixture shall be done under the environmental conditions specified in the protocol.

(3) If a vehicle is used to facilitate the mixing of a test substance with a carrier, assurance shall be provided that the vehicle does not interfere with the integrity of the test.

### § 792.232 Provisions applying to chemical fate studies.

All provisions contained in Part 792 apply to the chemical fate testing except the following:

Section 792.31 (c), (d), and (g)
Section 792.35 (b) and (c)
Section 792.43
Section 792.45
Section 792.47
Section 792.49
Section 792.81 (b)(1), (2), (6) through (9), and (12)
Section 792.90
Section 792.105 (a) through (d)
Section 792.113
Section 792.120(a) (5) through (13), and (16)
Section 792.185(a) (5) through (8), (10), (12), and (14)
Section 792.195 (c) and (d)
Section 792.228.

# PART 795—PROVISIONAL TEST GUIDELINES

## Subpart A—[Reserved]

## Subpart B—Provisional Chemical Fate Guidelines

Sec.
795.45 Inherent biogradability: Modified SCAS test for chemical substances that are water insoluble or water insoluble and volatile.
795.54 Anaerobic microbiological transformation rate data for chemicals in the subsurface environment.
795.70 Indirect photolysis screening test: Sunlight photolysis in waters containing dissolved humic substances.

## Subpart C—Provisional Environmental Effects Guidelines

795.120 Gammarid acute toxicity test.

## Subpart D—Provisional Health Effects Guidelines

795.223 Pharmacokinetic test.
795.225 Dermal pharmacokinetics of DGBE and DGBA.
795.230 Oral and inhalation pharmacokinetic test.
795.235 Toxicokinetic test.
795.250 Developmental neurotoxicity screen.
795.260 Subchronic oral toxicity test.
795.285 Morphologic transformation of cells in culture.

AUTHORITY: 15 U.S.C. 2603.

## Subpart A—[Reserved]

## Subpart B—Provisional Chemical Fate Guidelines

### § 795.45 Inherent biodegradability: Modified SCAS test for chemical substances that are water insoluble or water insoluble and volatile.

(a) *Introductory information*—(1) *Prerequisites.* (i) Water solubility of the test chemical must be established.

(ii) The organic carbon content of the test chemical must be established.

(2) *Guidance information.* (i) Information on the relative proportions of the major components of the test chemical will be useful in interpreting the results obtained.

(ii) Information on the toxicity of the chemical may be useful to the interpretation of low results and in the selection of appropriate test concentrations.

(3) *Standard documents.* This Test Guideline has been based on the papers cited under paragraphs (d) (1) and (2) of this section.

(b) *Method*—(1) *Introduction, purpose, scope, relevance, application and limits of test*—(i) *The method.* (A) The method is an adaptation of the Soap and Detergent Association Semi-Continuous Activated Sludge (SCAS) procedure for assessing the primary biodegradation of alkylbenzene sulphonate. The method involves exposure of the chemical to relatively high concentrations of microorganisms over a long time period (possibly several months). The viability of the microorganisms is maintained over this period by daily addition of a settled sewage feed.

(B) Since the conditions provided by the test are highly favorable to the selection and/or adaptation of microorganisms capable of degrading the test chemical, the procedure may also be used to produce microbial inocula adapted to selected chemicals for use in other tests. The test is applicable to organic chemicals that are water insoluble or water insoluble and volatile and that are not inhibitory to bacteria at the test concentration.

(ii) *Reference chemicals.* In some cases when investigating a new chemical, reference chemicals may be useful; however, specific reference chemicals cannot yet be recommended. Data on several chemicals used in interlaboratory tests are provided (see Table 1 in this paragraph) primarily so that calibration of the method may be performed from time to time and to permit comparison of results when another method is employed.

TABLE 1—EXAMPLES OF RESULTS OF SCAS TEST ON VARIOUS CHEMICALS USED IN THE OECD/EEC INTERLABORATORY TEST

| Test chemical | $O_T$ (mg/l) | $O_T$-$O_t$ (mg/l) | Percentage biodegradation bioelimination |
|---|---|---|---|
| 4-Acetylaminobenzene sulphonate | 17.2 | 2.0 | 85 |
| Tetrapropylenebenzene sulphonate | 17.3 | 8.4 | 51.4 |

TABLE 1—EXAMPLES OF RESULTS OF SCAS TEST ON VARIOUS CHEMICALS USED IN THE OECD/EEC INTERLABORATORY TEST—Continued

| Test chemical | $O_T$ (mg/l) | $O_t$-$O_c$ (mg/l) | Percentage biodegradation bioelimination |
|---|---|---|---|
| 4-Nitrophenol | 16.9 | 0.8 | 95.3 |
| Diethylene glycol | 16.5 | 0.2 | 98.8 |
| Aniline | 16.9 | 1.7 | 95.6 |
| Cyclopentane tetracarboxylate | 17.9 | 3.2 | 81.1 |

Duration of test is 40 days, except 120 days for cyclopentane tetracarboxylate.

(iii) *Principle of the test method.* (A) Activated sludge from a sewage treatment plant is placed in an aeration (SCAS) unit. The test chemical and settled domestic sewage are added, and the mixture is aerated for 23 hours. The aeration is then stopped, the sludge is allowed to settle, and the supernatant liquor is removed. The sludge remaining in the aeration chamber is then mixed with a further aliquot of test chemical and sewage and the cycle is repeated.

(B) This method requires use of a chemical-specific analytical technique or $^{14}$C-labeled test chemical. The purpose of the method is to determine the fate of the test chemical in a conventional activated sludge treatment plant. To this end, a complete mass balance for the test chemical is established by quantifying parent chemical in settled effluent sludge solids (insoluble test chemicals whether volatile or not), effluent plus solids (insoluble test chemicals whether volatile or not), and off gases (volatile test chemicals only). The identification and quantification of degradation products in all phases are recommended, but not required.

(iv) *Quality criteria*—(A) *Reproducibility.* When primary biodegradation is considered, very precise data are obtained for chemicals that are extensively degraded. The results reported in the reference under paragraph (d)(1) of this section suggest 95-percent confidence limits of less than ±3 percent, and this includes interlaboratory tests. As would be expected, wider confidence limits are obtained for less biodegradable chemicals.

(B) *Possibility of standardization.* Since the method uses a feed of settled sewage, absolute standardization is not possible unless this feed were replaced by synthetic sewage. However, since the method is designed to give an indication of the biodegradability potential of a chemical and is not a simulation test such standardization is unnecessary.

(C) *Possibility of automation.* Automation of this method would be possible but would be expensive. As the method is not labor intensive, the exercise would offer few advantages.

(2) *Description of the test procedure*—(i) *Preparations.* (A) The aeration units are cleaned and fixed in a suitable support. The air inlet tubes are connected to the supply manifold. A small laboratory-scale air compressor is used to aerate the units, and the air is presaturated with water to reduce evaporation losses from the units.

(B) If the test chemical is volatile, exhaust gases from the aeration units shall be passed through a suitable trap (such as Amberlite XAD-4, Rohm and Haas, Philadelphia, PA) to remove volatilized organics.

(C) A sample of mixed liquor from an activated sludge plant treating predominantly domestic sewage is obtained. Approximately 150 milliliters (ml) of the mixed liquor are required for each aeration unit.

(D) The organic carbon analyzer is calibrated using potassium hydrogen phthalate.

(E) Stock solutions of the test chemicals are prepared: The concentration normally required is 400 milligrams per liter (mg/L) as organic carbon which gives a test chemical concentration of 20 mg/L carbon at the start of each aeration cycle if no biodegradation is occurring.

(F) If the test chemical is insoluble in water at 400 mg/L it may be necessary to use ultrasound dispersion to obtain a uniform stable suspension. Alternatively, test chemical may be added directly to the aeration units.

(G) The organic carbon content of the stock solutions is measured.

(ii) *Test conditions.* A high concentration of aerobic microorganisms is used, and the effective detention period is 36 hours. The carbonaceous material in the sewage feed is oxidized extensively within 8 hours of the start of each aeration cycle. Thereafter, the sludge respires endogenously for the remainder of the aeration period, during which time the only available substrate is the test chemical unless this is also readily metabolized. These features, combined with daily reinoculation of the test when domestic sewage is used as the medium, provide highly favorable conditions for both adaptation and biodegradation.

(iii) *Performance of the test.* (A) A sample of mixed liquor from a suitable activated sludge plant is obtained and aerated during transportation to the laboratory. Each aeration unit is filled with 150 ml of mixed liquor, and the aeration is started. After 23 hours, aeration is stopped, and the sludge is allowed to settle for 45 minutes. The tap is opened, and 100 ml of the supernatant liquor is withdrawn. A sample of settled domestic sewage is obtained immediately before use, and 100 ml are added to the sludge remaining in each aeration unit. Aeration is started anew. At this stage no test chemicals are added, and the units are fed daily with domestic sewage only until a clear supernatant liquor is obtained on settling. This usually takes up to 2 weeks, by which time the dissolved organic carbon in the supernatant liquor at the end of each aeration cycle should be less than 12 mg/L.

(B) At the end of this period the individual settled sludges are mixed, and 50 ml of the resulting composite sludge are added to each unit.

(C) One hundred ml of settled sewage are added to the control units, and 95 ml of settled sewage plus 5 ml of the appropriate test chemical stock solution or suspension (400 mg organic carbon/L) to the test units. If test chemical is added directly to aeration units, 100 ml of settled sewage is added, as in the control units.

(D) Aeration is started again and continued for 23 hours. The sludge is then allowed to settle for 45 minutes and the supernatant drained off and analyzed for parent chemical. Before analysis the liquors are filtered through washed 0.45-micrometer membrane filters and certifuged. Temperature of the sample must not exceed 40 °C while it is in the centrifuge.

(E) If the test chemical is insoluble or expected to sorb significantly to sludge solids, settled sludge is also collected by an appropriate means (such as centrifugation) and extracted to remove test chemical, and the extract is analyzed for parent chemical.

(F) If the test chemical is volatile, traps for removing volatile organics from exhaust gases are also extracted and the extracts analyzed for parent chemical.

(G) The fill and draw procedure under paragraphs (b)(2)(iii) (C) through (F) of this section is repeated daily throughout the test.

(H) Before settling, it may be necessary to clean the walls of the units to prevent the accumulation of solids above the level of the liquid. A separate scraper or brush is used for each unit to prevent cross contamination.

(I) The length of the test for chemicals showing little or no biodegradation is indeterminate, but experience suggests that this should be at least 12 weeks.

(c) *Data and reporting*—(1) *Treatment of the results.* (i) The concentration of parent chemical in settled effluent sludge solids (insoluble test chemicals whether volatile or not), effluent plus solids (insoluble test chemicals whether volatile or not), and off-gases (volatile test chemicals only) is plotted versus time for the test units. As biodegradation is achieved the level of the test chemical will decrease and approach a steady state. Once the levels of the test chemical are found to be constant over three consecutive measurements, three further measurements are made.

(ii) An example of the application of specific analytical technique to the SCAS test is discussed in the reference in paragraph (d)(2) of this section.

(d) *Literature references.* For additional background information on this test guideline the following references should be consulted:

(1) "A Procedure and Standards for the Determination of the Biodegrada-

bility of Alkyl Benzene Sulphonate and Linear Alkylate Sulphonate", *Journal of the American Chemical Society*, 42:986, 1965.

(2) Games, L.M., King, J.E., and Larson, R.J. "Fate and distribution of a quaternary ammonium surfactant octadecyltrimethylammonium chloride (OTAC), in wastewater treatment." *Environmental Science and Technology*, 16:483–488, 1982.

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[52 FR 21026, June 4, 1987; 52 FR 32990, Sept. 1, 1987]

### § 795.54 Anaerobic microbiological transformation rate data for chemicals in the subsurface environment.

(a) *Introduction.* (1) This guideline describes laboratory methods for developing anaerobic microbiological transformation rate data for organic chemicals in subsurface materials. The method is based on a time-tiered approach. For chemicals that are degraded rapidly, only a portion (the 0, 4, and 8 week sampling periods, for example) of the test will have to be completed; however, for slowly degrading chemicals, the entire test may have to be performed (64 weeks). The data will be used to calculate degradation rate constants for each tested chemical over a range of environmental conditions. The rate constants obtained from testing will be integrated into algorithms to assess the fate of organic chemicals leaching into ground water from waste management facilities.

(2) Anaerobic transformations are evaluated under methanogenic and sulfur-reducing conditions. Aerobic biodegradation was not included in the modeling analysis for two reasons:

(i) Aerobic biodegradation would be limited by the concentration of oxygen in ground water. In the laboratory, oxygen would probably not be limiting, and the resulting degradation rates obtained would possibly be overestimations of actual subsurface degradation rates.

(ii) Aerobic degradation would only occur at the leading edge of a contaminant plume where dispersion and other processes dilute the plume with oxygenated water, as stated in Wilson et al. (1985), in paragraph (d)(24) of this section.

(3) The anaerobic transformation of chemicals in selected subsurface samples shall be estimated from subsurface microcosm studies using methods adapted from procedures recently reported by Wilson et al. (1986), in paragraph (d)(25) of this section. These procedures shall be used to determine the length of the adaptation period (time interval before detectable degradation of the chemical can be observed) and the half-life of the chemical following the adaptation period. Supporting laboratory methods shall be used to measure the levels of residual test chemical, intermediate degradation products, biomass, and other physical-chemical parameters.

(b) *Laboratory procedures*—(1) *Identification of subsurface sampling sites, collection of subsurface materials, and transportation and storage of subsurface materials.*—(i) A minimum of six subsurface sampling sites shall be identified on the basis of two temperatures and three pH values. Three of the sites shall have annual average temperatures near 10 °C, and three of the sites shall have temperatures near 20 °C. These values are chosen to represent the high and low temperatures commonly observed in aquifers and are one standard deviation on either side of the mean temperature of 15 °C. Generally, low temperature sites are located in northern latitude areas of the United States, and high temperatures correspond to southern latitude areas.

(ii) Acidic (pH 4.5 to 6.0), neutral (pH 6.5 to 7.5), and alkaline (pH 8.0 to 9.5) sites shall be selected for each temperature range. These ranges of pH values for ground waters are selected to estimate the effect of pH on microbial degradation capacity and to examine the effect of chemical form on the degradation of chemicals having dissociable hydrogen (i.e., degradation of the protonated and unprotonated forms of the chemical). Ground waters at all sites shall have dissolved-oxygen levels below 0.1 mg/L and sulfate concentrations below 10 mg/L.

(iii) Samples of subsurface materials shall be collected in a manner that protects them from contamination from surface materials and maintains anaerobic conditions. An appropriate procedure has been reported by Wilson et al. (1983), in paragraph (d)(26) of this section. First, a bore hole is drilled to the desired depth with an auger. Then the auger is removed and the sample taken with a wireline piston core barrel, as reported by Zapico et al., 1987, in paragraph (d)(14) of this section. The core barrel is immediately transferred to an anerobic chamber, filled and continually purged with nitrogen gas, and all further manipulations are performed in the chamber. Using aseptic procedures, up to 5 centimeters (cm) of the core is extruded, then broken off to produce an uncontaminated face. A sterile paring device is then installed, and the middle 30 to 35 cm of the core is extruded, paring away the outer 1.0 cm of core material. As a result, the material that had been in contact with the core barrel, and thus might be contaminated with surface microorganisms, is discarded. Modifications of this technique can be used for samples obtained from deep coring devices when auger equipment is insufficient because of the depth of the aquifer. Subsurface material shall be stored under nitrogen gas and on ice and shall be used in microcosm studies within 7 days of collection.

(iv) Ground waters will be collected from the bore hole used to collect subsurface materials. Ground waters will be pumped to the surface. The bore hole should be purged with argon before pumping begins. The pumping mechanism should be flushed with enough ground water to insure that a representative ground water sample is obtained. This flushing process generally requires a volume equal to 3 to 10 times the volume of water in the bore hole. Once flushing is complete, ground water samples should be collected, and stored under nitrogen and on ice for transport back to the laboratory. Ground waters shall be sterilized by filtration through 0.22 micrometer (μm) membranes on-site in a portable anaerobic chamber filled and continually purged with nitrogen gas. The sterile water shall be stored under nitrogen and on ice, and shall be used in microcosm studies within 7 days of collection.

(v) Two samples shall be collected from each of the 6 sites. Each core sample shall be assayed for test chemical degradation and analyzed for biomass (heterotrophic, sulfate-reducing, and methanogenic) and physical-chemical parameters (pH, cation exchange capacity, total organic carbon, percent base saturation, percent silt, percent sand, percent clay, redox potential, percent ash-free dry weight). Each corresponding ground water sample will be analyzed for pH, dissolved oxygen, dissolved organic carbon, nutrients (sulfate, phosphate, nitrate), conductivity, and temperature.

(2) *Anaerobic Microcosm assay.* (i) Microcosms shall consist of 160-milliliter (mL) serum bottles which have been filled completely with a slurry of subsurface material and ground water (20 grams equivalent dry wt (oven dry wt. 103 °C) solid to 80 mL ground water). One series of serum bottles shall be amended to a level of 200 mg/L sulfate (weight/volume added as sodium sulfate) to stimulate sulfate-reducing conditions. If the level of soluble sulfate falls below 50 mg/L at any sampling time, additional sulfate (200 mg/L, weight/volume) should be added to all remaining sulfate-amended microcosms. Soluble sulfate levels should be measured by the method of Watwood et al. (1986), in paragraph (d)(23) of this section. A second series shall be left unamended to simulate methanogenic conditions. All manipulations in preparing the microcosms shall be performed aseptically under strict anaerobic conditions, as described in Kaspar and Tiedje (1982) in paragraph (d)(10) of this section, or other equivalent methods, and all equipment in contact with the subsurface samples shall be sterilized. Sterile controls shall be prepared by autoclaving the samples for a minimum of 1 hour on each of 3 consecutive days. Test chemical amendments shall be prepared in sterile nitrogen-purged ground water. Sparingly soluble and volatile chemicals shall be added to sterile, nitrogen-purged ground water and then stirred overnight without a head space.

(ii) The active and control microcosms shall be dosed with the test chemical and 0.0002 percent (w/v) Resasurin as a redox indicator, and then each unit shall be immediately sealed with a Teflon®-coated gray butyl rubber septum and crimp seal. As stated previously, all manipulations shall be performed under strict anaerobic conditions, as described in Kaspar and Tiedje (1982) in paragraph (d)(10) of this section, or other equivalent methods. The microcosms shall be stored in the dark at the original in-situ temperature. Active microcosms and control microcosms, randomly selected from the sulfate-amended series and the unamended series, shall be sacrificed and analyzed at 0, 4, 8, 16, 32, and 64 weeks for residual test chemical and the formation of degradation intermediates. Once the residual level of the chemical drops below 5 percent of the initial concentration, analysis of microcosms at subsequent time periods is not required. The active microcosms and control microcosms from both series, at weeks 0, 16, and 64 (or randomly selected from the remaining samples the week following 95 percent degradation of the chemical, if less than week 64) shall also be analyzed for heterotrophic, sulfate-reducing, and methanogenic bacteria.

(iii) Three concentrations of each chemical tested shall be used. The test chemical concentrations should range between a low level of 30 times the health-based level and a level that equates to the chemical's solubility (or to a level that causes inhibition of the test chemical's degradation).

(iv) Biomass measurements shall be made for heterotrophic, sulfate-reducing, and methanogenic bacteria. Biomass measurements have been included to insure comparability of results between samples of subsurface materials. Degradation rates derived from sediment samples having significantly high or low (student "t" test, 90 percent level) bacterial populations would not be considered in subsequent modeling efforts. Also, the ratio of sulfate-reducing organisms to methanogenic organisms would be used to determine if the dominant redox conditions were sulfate-reducing or methanogenic. Anaerobic techniques described by Kaspar and Tiedje (1982), cited in paragraph (d)(10) of this section, or other equivalent methods, shall be used.

(v) Heterotrophic bacterial concentrations shall be measured by a modification of the procedure developed by Molongoski and Klug (1976) and Clark (1965), cited in paragraphs (d)(13) and (d)(6) of this section, respectively. A ten-mL sample taken from the center of the appropriate microcosm, which has been well mixed, shall be aseptically transferred to 100 mL of sterile dilution medium and agitated to suspend the organisms. Ten-mL samples shall then be transferred immediately from the center of the suspension to a 90-mL sterile dilution medium blank to give a $10^{-2}$ dilution; 10 mL shall be similarly transferred to another 90-mL of sterile dilution medium to obtain a dilution of $10^{-3}$. This process shall be repeated to give a dilution series through at least $10^{-7}$. Only the $10^{-1}$ dilution need be prepared from control samples. The dilution series can be modified to include dilutions of greater than $10^{-7}$, if necessary, and if sufficient sample is available. From the highest dilution, 0.1-mL portions shall be transferred to the surface of each of three dilute tryptone glucose extract agar plates. The sample shall be spread immediately over the surface of the plates; the process shall be repeated for lower dilutions. Dilute tryptone glucose agar plates shall be prepared by combining 24.0 g tryptone glucose extract agar in 1 liter of distilled water. The mixture shall be autoclaved, and 25 mL of the molten agar shall be transferred to petri plates. Agar plates should be stored in an anaerobic chamber for a minimum of 24 hours before use. The inoculated plates shall be incubated in plastic bags in the glove box, or, if necessary, removed and kept in anaerobic jars. After 14 days of incubation, the plates shall be examined and the total count per gram of dry sediment material shall be determined. If the plates from the most dilute sample show more than 300 colonies, the dilution series was inadequate. In this case, all of the plates shall be discarded, and the process shall be repeated with greater dilutions, as appropriate.

(vi) Sulfate-reducing species shall be enumerated by the MPN (most probable number) technique as descibed in Pankhurst (1971) in paragraph (d)(15) of this section, or other equivalent method. The dilution series shall be prepared as described for heterotrophic bacteria.

(vii) Methanogenic bacteria shall be enumerated by the MPN technique as described by Jones et al. (1982) in paragraph (d)(9) of this section, or by another equivalent method. The dilution series shall be prepared as described for heterotrophic bacteria.

(3) *Analytical measures of the loss of test chemical and intermediate degradation products.* (i) The loss of test chemical shall be quantified by measuring the residual test chemical. The formation of degradation intermediates shall be quantified in microcosm assays for test chemicals that can potentially be transformed. Analysis for degradation intermediates shall be required when the level of test chemical has been reduced by more than 25 percent. Concentrations of the potential degradation products 1,2-, 1,3-, and 1,4-dichlorobenzene, and 1,2,4,5-tetrachlorobenzene shall be measured in the appropriate microcosms used to analyze the degradation of pentachlorobenzene. The concentration of the potential degradation product dibromomethane shall be measured in the appropriate microcosms used to analyze the degradation of bromoform. The potential degradation products methanethiol and chloromethane (methyl chloride) shall be measured in the appropriate microcosm used to analyze the degradation of trichloromethanethiol. The potential intermediate products 1,2-, 1,3-, and 1,4-dichlorobenzene shall be measured in the appropriate microcosm used to analyze the degradation of 1,2,4,5-tetrachlorobenzene.

(ii) Measurements of test chemical and intermediate degradation products will require organic analytical techniques tailored to the specific test chemical and subsurface material being investigated. Several extraction and purge-trap techniques are available for the recovery of residual test chemicals and degradative intermediates from subsurface materials. Unique analytical procedures would have to be developed or modified for each test chemical and sediment. The following represent examples of such techniques:

(A) Soxlet extraction as described in Anderson et al. (1985), Bossart et al. (1984), Eiceman et al. (1986), Grimalt et al. (1984), and Kjolholt (1985) in paragraphs (d) (2), (3), (7), (8), and (11) of this section, respectively.

(B) Shake flask method as described in Brunner et al. (1985), and Russel and McDuffie (1983) in paragraphs (d) (4) and (16) of this section, respectively.

(C) Sonification as described in Schellenberg et al. (1984) in paragraph (d)(17) of this section.

(D) Homogenization as described in Fowlie and Sulman (1986), Lopez-Avila et al. (1983), Sims et al. (1982), Stott and Tabatabai (1985), and U.S. EPA (1982) in paragraphs (d) (5), (12), (18), (19), and (22) of this section, respectively.

(E) Purge-trap techniques have been described by Wilson et al. (1986) in paragraph (d)(24) of this section.

(iii) These procedures can be readily coupled to gas chromatography (GC) and high-pressure liquid chromatography (HPLC) procedures to quantify the chemicals of interest. Whatever analytical procedure is selected shall follow Good Laboratory Practice Standards of 40 CFR Part 792.

(4) *Characterization of subsurface materials and ground waters.* (i) Subsurface materials shall be classified, described, and characterized as to soil type and physical and chemical properties using standard procedures as described by the Soil Conservation Service (U.S. Department of Agriculture, 1972 and 1975) in paragraphs (d) (20) and (21) of this section, or other equivalent methods. Ten parameters will be measured as follows:

(A) Total organic carbon (TOC).
(B) pH.
(C) Cation exchange capacity.
(D) Percent base saturation.
(E) Percent silt.
(F) Percent sand.
(G) Percent clay.
(H) Redox potential.
(I) Percent ash-free dry weight.
(J) Texture.

(ii) Ground water shall be characterized for the following, by standard water and wastewater methods described by the American Public Health Association (1985) in paragraph (d)(1) of this section, or other equivalent methods:

(A) pH.

(B) Dissolved oxygen.

(C) Dissolved organic carbon.

(D) Nutrients including sulfate, phosphate, and nitrate.

(E) Conductivity.

(F) Temperature.

(iii) The properties of pH, dissolved oxygen, and temperature shall be measured at the site of collection. All other properties shall be measured in the laboratory.

(c) *Data to be reported to the Agency.* Data shall be reported for the two subsurface samples and corresponding ground waters taken from the six different sampling sites.

(1) The following shall be reported for subsurface sediment samples:

(i) Levels of residual test chemicals (mg/gm/dry wt) quantified in each of the randomly selected replicate microcosm and sterile controls at the specific time periods identified under the anaerobic microcosm assay.

(ii) Numbers of heterotrophic, sulfate-reducing, and methanogenic bacteria (colony forming units (CFU) or most probable number units (MPNU) per gm dry wt) enumerated in each replicate microcosm and sterile controls at the specific time periods identified under the anaerobic microcosm assay.

(iii) Levels of persistent degradation intermediates identified in microcosm and sterile controls at the specific time periods identified under the anaerobic microcosm assay.

(iv) Measured values for pH, cation exchange capacity (meg/100 gm dry wt), percent base saturation, percent silt (percent dry wt), percent sand (percent dry wt), percent clay (percent dry wt), redox potential (Eh, Standard Hydrogen Electrode), percent ash free dry weight (percent dry wt), and a description of texture.

(2) For ground water samples, the analysis report shall provide measured values for:

(i) pH.

(ii) Dissolved oxygen (mg/L).

(iii) Dissolved organic carbon (mg/L).

(iv) Nutrients including sulfate (mg/L), phosphate (mg/L), and nitrate (mg/L).

(v) Conductivity (umho, 25 °C).

(vi) Temperature (°C).

(d) *References.* For additional background information cited in this protocol, the following references should be consulted:

(1) American Public Health Association, American Water Works Association, and Water Pollution Control Federation. "Standard methods for the examination of water and wastewater," 16th ed., A.E. Greenberg, R.R. Trussel, and L.C. Clesceri (eds.), American Public Health Association, Washington, DC (1985).

(2) Anderson, J.W., G.H. Herman, D.R. Theilen, and A.F. Weston. "Method verification for determination of tetrachlorodibenzodioxine in soil." *Chemosphere* 14:1115-1126 (1985).

(3) Bossart, I., W.M. Kachal, and R. Bartha. "Fate of hydrocarbons during oil sludge disposal in soil." *Applied and Environmental Microbiology* 47:763-767 (1984).

(4) Brunner, W., F.H. Sutherland, and D.D. Focht. "Enhanced biodegradation of polychlorinated biphenyls in soil by analog enrichment and bacterial inoculation." *Journal of Environmental Quality* 14:324-328 (1985).

(5) Fowlie, P.J.A., and T.L. Bulman. "Extraction of anthracene and benzo(a)-pyrene from soil." *Analytical Chemistry* 58:721-723 (1986).

(6) Clark, F.E. "Agar-plate method for total microbial count," p. 1460-1466. In: C.A. Black (ed.), "Methods of soil analysis. Part 2. Chemical and Microbiological Properties." American Society of Agronomy, Inc., Madison WI (1965).

(7) Eiceman, G.A., B. Davani, and J. Ingram. "Depth profiles for hydrocarbons and polycyclic aromatic hydrocarbons in soil beneath waste disposal pits from natural gas production." *Environmental Science and Technology.* 20:508-514 (1986).

(8) Grimalt, J., C. Marfil, and J. Albaiges. "Analysis of hydrocarbons in aquatic sediments." *International*

*Journal of Environmental Analytical Chemistry* 18:183–194 (1984).

(9) Jones, J.G., B.M. Simon, and S. Gardener. "Factors affecting methanogenesis and associated anaerobic processes in the sediments of a stratified eutrophic lake." *Journal of General Microbiology* 128:1-11 (1982).

(10) Kaspar, H.F., and J.M. Tiedje. "Anaerobic bacteria and processes," p. 989-1009. In: A.L. Page (ed.), "Methods of Soil Analysis. Part 2. Chemical and Microbiological Properties." American Society of Agronomy, Madison, WI (1982).

(11) Kjolholt, J. "Determination of trace amounts of organophosphorous pesticides and related compounds in soils and sediments using capillary gas chromatography and a nitrogen-phosphorous detector." *Journal of Chromatography* 325:231–238 (1985).

(12) Lopez-Avila, V., R. Northcutt, J. Onstot, M. Wickham, and S. Billets. "Determination of 51 priority organic compounds after extraction from standard reference materials." *Analytical Chemistry* 55:881–889 (1983).

(13) Molongoski, J.J., and M.J. Klug. "Characterization of anaerobic heterotrophic bacteria isolated from freshwater lake sediments." *Applied Environmental Microbiology* 31:83–90 (1976).

(14) Zapico, M.M., S. Vales, and J.S. Cherry. "A wireline piston core barrel for sampling cohesionless sand and gravel below the water table." *Ground Water Monitoring Review.* Summer, Vol. 7, No. 3:74–82 (1987).

(15) Pankhurst, E.S. "The isolation and enumeration of sulphate-reducing bacteria," p. 223-240. In: D.A. Shapton and R.G. Board (eds.), "Isolation of Anaerobes." Academic Press, Inc., New York (1971).

(16) Russell, D.J., and B. McDuffie. "Analysis for phthalate esters in environmental samples: Separation from PCBs and pesticides using dural column chromatography." *International Journal of Environmental Analytical Chemistry* 15:165–183 (1983).

(17) Schellenberg, K., C.L. Leuenberger, and R.P. Schwarzenback. "Sorption of chlorinated phenols by natural sediments and aquifer materials." *Environmental Science and Technology.* 18:652–657 (1984).

(18) Sims, R.C. "Land treatment of polynuclear wastes." Ph.D. dissertation. North Carolina State University, Raleigh, NC (1982).

(19) Stott, D.E., and M.A. Tabatabai. "Identification of phospholipids in soils and sewage sludges by high-performance liquid chromatography." *Journal of Environmental Quality.* 14:107-110 (1985).

(20) United States Department of Agriculture. "Soil survey laboratory methods and procedures for collecting soil samples." *Soil Survey Investigations Report No. 1.* Soil Conservation Service. Soil Survey Investigation (1972).

(21) United States Department of Agriculture. "Soil taxonomy: a basic system of soil classification for making and interpreting soil surveys." *Agricultural Handbook 436.* Soil Conservation Service (1975).

(22) U.S. Environmental Protection Agency. "Test Methods for Evaluating Solid Wastes: Physical/Chemical Methods." Second Edition. SW-846. U.S. Environmental Protection Agency, Washington, DC (1982).

(23) Watwood, M.E., J.W. Fitzgerald, and J.R. Gosz. Sulfur processing in forest soil and litter along an elevational and vegetative gradient. *Canadian Journal of Forest Resources.* 16:689–695 (1986).

(24) Wilson, J.T., J.F. McNabb, J.W. Cochran, T.H. Wang, M.B. Tomson, and P.B. Bedient. "Influence of microbial adaptation of the fate of organic pollutants in ground water." *Environmental Toxicology and Chemistry.* 4:721-726 (1985).

(25) Wilson, B.H., G.B. Smith, and J.F. Rees. "Biotransformation of selected alkylbenzenes and halogenated aliphatic hydrocarbons in methanogenic aquifer material: A microcosm study." *Environmental Science and Technology.* 20:997-1002 (1986).

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**[53 FR 22320, June 15, 1988]**

### § 795.70 Indirect photolysis screening test: Sunlight photolysis in waters containing dissolved humic substances.

(a) *Introduction.* (1) Chemicals dissolved in natural waters are subject to two types of photoreaction. In the first case, the chemical of interest absorbs sunlight directly and is transformed to products when unstable excited states of the molecule decompose. In the second case, reaction of dissolved chemical is the result of chemical or electronic excitation transfer from light-absorbing humic species in the natural water. In contrast to direct photolysis, this photoreaction is governed initially by the spectroscopic properties of the natural water.In general, both indirect and direct processes can proceed simultaneously. Under favorable conditions the measurement of a photoreaction rate constant in sunlight ($K_{pE}$) in a natural water body will yield a net value that is the sum of two first-order reaction rate constants for the direct ($k_{DE}$) and indirect ($k_{IE}$) pathways which can be expressed by the relationship

*Equation 1*

$$k_{pE}=k_{DE}+k_{IE}.$$

This relationship is obtained when the reaction volume is optically thin so that a negligible fraction of the incident light is absorbed and is sufficiently dilute in test chemical; thus the direct and indirect photoreaction processes become first-order.

(3) In pure water only, direct photoreaction is possible, although hydrolysis, biotransformation, sorption, and volatilization also can decrease the concentraton of a test chemical. By measuring $k_{pE}$ in a natural water and $k_{DE}$ in pure water, $k_{IE}$ can be calculated.

(4) Two protocols have been written that measure $k_{DE}$ in sunlight or predict $k_{DE}$ in sunlight from laboratory measurements with monochromatic light (USEPA (1984) under paragraph (f)(14) and (15) of this section; Mill et al. (1981) under paragraph (f)(9) of this section; Mill et al. (1982) under paragraph (f)(10) of this section; Mill et al. (1983) under paragraphs (f)(11) of this section). As a preface to the use of the present protocol, it is not necessary to know $k_{DE}$; it will be determined under conditions that definitively establish whether $k_{IE}$ is significant with respect to $k_{DE}$.

(5) This protocol provides a cost effective test method for measuring $k_{IE}$ for test chemicals in a natural water (synthetic humic water, SHW) derived from commercial humic material. It describes the preparation and standardization of SHW. To implement the method, a test chemical is exposed to sunlight in round tubes containing SHW and tubes containing pure water for defined periods of time based on a screening test.

(6) To correct for variations in solar irradiance during the reaction period, an actinometer is simultaneously insolated. From these data, an indirect photoreaction rate constant is calculated that is applicable to clear-sky, near-surface, conditions in fresh water bodies.

(7) In contrast to $k_{DE}$, which, once measured, can be calculated for different seasons and latitudes, $k_{IE}$ only applies to the season and latitude for which it is determined. This condition exists because the solar action spectrum for indirect photoreaction in humic-containing waters is not generally known and would be expected to change for different test chemicals. For this reason, $k_{pE}$, which contains $k_{IE}$, is likewise valid only for the experimental data and latitude.

(8) The value of $k_{pE}$ represents an atypical quantity because $k_{IE}$ will change somewhat from water body to water body as the amount and quality of dissolved aquatic humic substances change. Studies have shown, however, that for optically-matched natural waters, these differences are usually within a factor of two (Zepp et al. (1981) under paragraph (f)(17) of this section).

(9) This protocol consists of three separate phases that should be completed in the following order: In Phase 1, SHW is prepared and adjusted; in Phase 2, the test chemical is irradiated in SHW and pure water (PW) to obtain approximate sunlight photoreaction rate constants and to determine whether direct and indirect photoprocesses are important; in Phase 3, the test chemical is again irradiated in PW and SHW. To correct for photob-

leaching of SHW and also solar irradiance variations, tubes containing SHW and actinometer solutions are exposed simultaneously. From these data $k_{pE}$ is calculated that is the sum of $k_{IE}$ and $k_{DE}$ (Equation 1) (Winterle and Mill (1985) under paragraph (f)(12) of this section).

(b) *Phase 1—Preparation and standardization of synthetic natural water*—(1) *Approach.* (i) Recent studies have demonstrated that natural waters can promote the indirect (or sensitized) photoreaction of dissolved organic chemicals. This reactivity is imparted by dissolved organic material (DOM) in the form of humic substances. These materials absorb sunlight and produce reactive intermediates that include singlet oxygen ($^1O_2$) (Zepp et al. (1977) under paragraph (f)(20) of this section, Zepp et al. (1981) under paragraph (f)(17) of this section, Zepp et al. (1981) under paragraph (f)(18) of this section, Wolff et al. (1981) under paragraph (f)(16) of this section, Haag et al. (1984) under paragraph (f)(6) of this section, Haag et al. (1984) under paragraph (f)(7) of this section); peroxy radicals ($RO_2\cdot$) (Mill et al. (1981) under paragraph (f)(9) of this section; Mill et al. (1983) under paragraph (f)(8) of this section); hydroxyl radicals ($HO\cdot$) (Mill et al. (1981) under paragraph (f)(9) of this section, Draper and Crosby (1981, 1984) under paragraphs (f)(3) and (4) of this section); superoxide anion ($O_2^{-}\cdot$) and hydroperoxy radicals ($HO\cdot$), (Cooper and Zika (1983) under paragraph (f)(1) of this section, Draper and Crosby (1983) under paragraph (f)(2) of this section); and triplet excited states of the humic substances (Zepp et al. (1981) under paragraph (f)(17) of this section, Zepp et al. (1985) under paragraph (f)(21) of this section). Synthetic humic waters, prepared by extracting commercial humic or fulvic materials with water, photoreact similarly to natural waters when optically matched (Zepp et al. (1981) under paragraphs (f)(17) and (18) of this section).

(ii) The indirect photoreactivity of a chemical in a natural water will depend on its response to these reactive intermediates, and possibly others yet unknown, as well as the ability of the water to generate such species. This latter feature will vary from water-to-water in an unpredictable way, judged by the complexity of the situation.

(iii) The approach to standardizing a test for indirect photoreactivity is to use a synthetic humic water (SHW) prepared by water-extracting commercial humic material. This material is inexpensive, and available to any laboratory, in contrast to a specific natural water. The SHW can be diluted to a dissolved organic carbon (DOC) content and uv-visible absorbance typical of most surface fresh waters.

(iv) In recent studies it has been found that the reactivity of SHW mixtures depends on pH, and also the history of sunlight exposure (Mill et al. (1983) under paragraph (f)(11) of this section). The SHW solutions initially photobleach with a time-dependent rate constant. As such, an SHW test system has been designed that is buffered to maintain pH and is pre-aged in sunlight to produce, subsequently, a predictable bleaching behavior.

(v) The purpose of Phase 1 is to prepare, pre-age, and dilute SHW to a standard mixture under defined, reproducible conditions.

(2) *Procedure.* (i) Twenty grams of Aldrich humic acid are added to a clean 2-liter Pyrex Erlenmeyer flask. The flask is filled with 2 liters of 0.1 percent NaOH solution. A stir bar is added to the flask, the flask is capped, and the solution is stirred for 1 hour at room temperature. At the end of this time the dark brown supernatant is decanted off and either filtered through coarse filter paper or centrifuged and then filtered through 0.4 )m microfilter. The pH is adjusted to 7.0 with dilute $H_2SO_4$ and filter sterilized through a 0.2 )m filter into a rigorously cleaned 2-liter Erlenmeyer flask. This mixture contains roughly 60 ppm DOC and the absorbance (in a 1 cm path length cell) is approximately 1.7 at 313 nm and 0.7 at 370 nm.

(ii) Pre-aging is accomplished by exposing the concentrated solution in the 2-liter flask to direct sunlight for 4 days in early spring or late fall; 3 days in late spring, summer, or early fall. At this time the absorbance of the solution is measured at 370 nm, and a dilution factor is calculated to decrease the absorbance to 0.50 in a 1 cm path length cell. If necessary, the pH is readjusted to 7.0. Finally, the mixture is brought to exact dilution with a precalculated volume of reagent-grade water to give a final absorbance of 0.500 in a 1-cm path length cell at 370 nm. It is tightly capped and refrigerated.

(iii) This mixture is SHW stock solution. Before use it is diluted 10-fold with 0.010 M phosphate buffer to produce a pH 7.0 mixture with an absorbance of 5.00 x $10^{-2}$ at 370 nm, and a dissolved organic carbon of about 5 ppm. Such values are characteristic of many surface fresh waters.

(3) *Rationale.* The foregoing procedure is designed to produce a standard humic-containing solution that is pH controlled, and sufficiently aged that its photobleaching first-order rate constant is not time dependent. It has been demonstrated that after 7 days of winter sunlight exposure, SHW solutions photobleached with a nearly constant rate constant (Mill et al. (1983) under paragraph (f)(11) of this section).

(c) *Phase 2—Screening test*—(1) *Introduction and purpose.* (i) Phase 2 measurements provide approximate solar photolysis rate constants and half-lives of test chemicals in PW and SHW. If the photoreaction rate in SHW is significantly larger than in PW (factor of > 2X) then the test chemical is subject to indirect photoreaction and Phase 3 is necessary. Phase 2 data are needed for more accurate Phase 3 measurements, which require parallel solar irradiation of actinometer and test chemical solutions. The actinometer composition is adjusted according to the results of Phase 2 for each chemical, to equalize as much as possible photoreaction rate constants of chemical in SHW and actinometer.

(ii) In Phase 2, sunlight photoreaction rate constants are measured in round tubes containing SHW and then mathematically corrected to a flat water surface geometry. These rate constants are not corrected to clear-sky conditions.

(2) *Procedure.* (i) Solutions of test chemicals should be prepared using sterile, air-saturated, 0.010 M, pH 7.0 phosphate buffer and reagent-grade (or purer) chemicals.[1] Reaction mixtures should be prepared with chemicals at concentrations at less than one-half their solubility in pure water and at concentrations such that, at any wavelengths above 290 nm, the absorbance in a standard quartz sample cell with a 1-cm path length is less than 0.05. If the chemicals are too insoluble in water to permit reasonable handling or analytical procedures, 1-volume percent acetonitrile may be added to the buffer as a cosolvent.

(ii) This solution should be mixed 9.00:1.00 by volume with PW or SHW stock solution to provide working solutions. In the case of SHW, it gives a ten-fold dilution of SHW stock solution. Six mL aliquots of each working solution should then be transferred to separate 12 × 100 mm quartz tubes with screw tops and tightly sealed with Mininert valves.[2] Twenty four tubes are required for each chemical solution (12 samples and 12 dark controls), to give a total of 48 tubes.

(iii) The sample tubes are mounted in a photolysis rack with the tops facing geographically north and inclined 30° from the horizontal. The rack should be placed outdoors over a black background in a location free of shadows and excessive reflection.

(iv) Reaction progress should be measured with an analytical technique that provides a precision of at least ±5 percent. High pressure liquid chromatography (HPLC) or gas chromatograph (GC) have proven to be the most general and precise analytical techniques.

(v) Sample and control solution concentrations are calculated by averaging analytical measurements for each solution. Control solutions should be analyzed at least twice at zero time and at other times to determine whether any loss of chemical in controls or samples has occurred by some adventitious process during the experiment.

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[1] The water should be ASTM Type IIA, or an equivalent grade.

[2] Mininert Teflon sampling vials are available from Alltech Associates, Inc., 202 Campus Dr., Arlington Heights, IL 60004.

(vi) Whenever possible the following procedures should be completed in clear, warm, weather so that solutions will photolyze more quickly and not freeze.

(A) Starting at noon on day zero, expose to sunlight 24 sample tubes mounted on the rack described above. Tape 24 foil-wrapped controls to the bottom of the rack.

(B) Analyze two sample tubes and two unexposed controls in PW and SHW for chemical at 24 hours. Calculate the round tube photolysis rate constants $(k_p)_{SHW}$ and $(k_p)_W$ if the percent conversions are J 20 percent but F 80 percent. The rate constants $(k_p)_{SHW}$ and $(k_p)_W$ are calculated, respectively, from Equations 2 and 3:

*Equation 2*

$(k_p)_{SHW} = (1/t)Pn(C_o/C_t)_{SHW}$ (in d$^{-1}$)

*Equation 3*

$(k_p)_W = (1/t)Pn(C_o/C_t)_W$ (in d$^{-1}$),

where the subscript identifies a reaction in SHW or PW; t is the photolysis time in calendar days; $C_o$ is the initial molar concentration; and $C_t$ is the molar concentration in the irradiated tube at t. In this case $t = 1$ day.

(C) If less than 20 percent conversion occurs in SHW in 1 day, repeat the procedure for SHW and PW at 2 days, 4 days, 8 days, or 16 days, or until 20 percent conversion is reached. Do not extend the experiment past 16 days. If less than 20 percent photoreaction occurs in SHW at the end of 16 days the chemical is "photoinert". Phase 3 is not applicable.

(D) If more than 80 percent photoreaction occurs at the end of day 1 in SHW, repeat the experiment with eight each of the remaining foil-wrapped PW and SHW controls. Divide these sets into four sample tubes each, leaving four foil-wrapped controls taped to the bottom of the rack.

(*1*) Expose tubes of chemical in SHW and PW to sunlight starting at 0900 hours and remove one tube and one control at 1, 2, 4, and 8 hours. Analyze all tubes the next day.

(*2*) Extimate $(k_p)_{SHW}$ for the first tube in which photoreaction is J 20 percent but F 80 percent. If more than 80 percent conversion occurs in the first SHW tube, report: "The half-life is less than one hour" and end all testing. The chemical is "photolabile." Phase 3 is not applicable.

(*3*) The rate constants $(k_p)_{SHW}$ and $(k_p)_W$ are calculated from equations 2 and 3 but the time of irradiation must be adjusted to reflect the fact that day-averaged rate constants are approximately one-third of rate constants averaged over only 8 daylight hours. For 1 hour of insolation enter $t = 0.125$ day into equation 2. For reaction times of 2, 4, and 8 hours enter 0.25, 0.50 and 1.0 days, respectively. Proceed to Phase 3 testing.

(*4*) Once $(k_p)_{SHW}$ and $(k_p)_W$ are measured, determine the ratio R from equation 4:

*Equation 4*

$R = (k_p)_{SHW}/(k_p)_W$.

The coefficient R, defined by Equation 4, is equal to $[(k_I + k_D)/k_D]$. If R is in the range 0 to 1, the photoreaction is inhibited by the synthetic humic water and Phase 3 does not apply. If R is in the range 1 to 2, the test chemical is marginally susceptable to indirect photolysis. In this case, Phase 3 studies are optional. If R is greater than 2, Phase 3 measurements are necessary to measure $k_{pE}$ and to evaluate $k_{IE}$.

(vii) Since the rate of photolysis in tubes is faster than the rate in natural water bodies, values of near-surface photolysis rate constants in natural and pure water bodies, $k_{pE}$ and $k_{DE}$, respectively, can be obtained from $(k_p)_{SHW}$ and $(k_p)_W$ from Equations 5 and 6:

*Equation 5*

$k_{pE} = 0.45(k_p)_{SHW}$

*Equation 6*

$k_{DE} = 0.45(k_p)_W$.

The factor 0.45 is an approximate geometric correction for scattered light in tubes versus horizontal surfaces. A rough value of $k_{IE}$, the rate constant for indirect photolysis in natural waters or SHW, can be estimated from the difference between $k_{pE}$ and $k_{DE}$ using Equation 7:

*Equation 7*

$k_{IE}=k_{pE}-k_{DE}$.

(3) *Criteria for Phase 2.* (i) If no loss of chemical is found in dark control solutions compared with the analysis in tubes at zero time (within experimental error), any loss of chemical in sunlight is assumed to be due to photolysis, and the procedure provides a valid estimate of $k_{pE}$ and $k_{DE}$. Any loss of chemical in the dark-control solutions may indicate the intervention of some other loss process such as hydrolysis, microbial degradation, or volatilization. In this case, more detailed experiments are needed to trace the problem and if possible eliminate or minimize the source of loss.

(ii) Rate constants determined by the Phase 2 protocol depend upon latitude, season, and weather conditions. Note that $(k_p)_{SHW}$ and $k_D$ values apply to round tubes and $k_{pE}$ and $k_{DE}$ values apply to a natural water body. Because both $(k_p)_{SHW}$ and $k_D$ are measured under the same conditions the ratio $((k_p)_{SHW}/k_D)$ is a valid measure of the susceptibility of a chemical to indirect photolysis. However, since SHW is subject to photobleaching, $(k_p)_{SHW}$ will decrease with time because the indirect rate will diminish. Therefore, R >2 is considered to be a conservative limit because $(k_p)_{SHW}$ will become systematically smaller with time.

(4) *Rationale.* The Phase 2 protocol is a simple procedure for evaluating direct and indirect sunlight photolysis rate constants of a chemical at a specific time of year and latitude. It provides a rough rate constant for the chemical in SHW that is necessary for Phase 3 testing. By comparison with the direct photoreaction rate constant, it can be seen whether the chemical is subject to indirect photoreaction and whether Phase 3 tests are necessary.

(5) *Scope and limitations.* (i) Phase 2 testing separates test chemicals into three convenient categories: "Photolabile", "photoinert", and those chemicals having sunlight half-lives in round tubes in the range of 1 hour to 50 days. Chemicals in the first two categories fall outside the practical limits of the test, and cannot be used in Phase 3. All other chemicals are suitable for Phase 3 testing.

(ii) The test procedure is simple and inexpensive, but does require that the chemical dissolve in water at sufficient concentrations to be measured by some analytical technique but not have appreciable absorbance in the range 290 to 825 nm. Phase 2 tests should be done during a clear-sky period to obtain the best results. Testing will be less accurate for chemicals with half-lives of less than 1 day because dramatic fluctuations in sunlight intensity can arise from transient weather conditions and the difficulty of assigning equivalent reaction times. Normal diurnal variations also affect the photolysis rate constant. Phase 3 tests should be started as soon as possible after the Phase 2 tests to ensure that the $(k_p)_{SHW}$ estimate remains valid.

(6) *Illustrative Example.*. (i) Chemical A was dissolved in 0.010 M pH 7.0 buffer. The solution was filtered through a 0.2 )m filter, air saturated, and analyzed. It contained $1.7\times10^{-5}$ M A, five-fold less than its water solubility of $8.5\times10^{-5}$ M at 25°C. A uv spectrum (1-cm path length) versus buffer blank showed no absorbance greater than 0.05 in the wavelength interval 290 to 825 nm, a condition required for the Phase 2 protocol. The 180 mL mixture was diluted by the addition of 20 mL of SHW stock solution.

(ii) The SHW solution of A was photolyzed in sealed quartz tubes (12×100 mm) in the fall season starting on October 1. At the end of 1 and 2 days, respectively, the concentration of A was found to be $1.13\times10^{-5}$ M and $0.92\times10^{-5}$ M compared to unchanged dark controls ($1.53\times10^{-5}$ M).

(iii) The tube photolysis rate constant of chemical A was calculated from Equation 2 under paragraph (c)(2)(vi)(B) of this section. The first time point at day 1 was used because the fraction of A remaining was in the range 20 to 80 percent:

$$(k_p)_{SHW}=(1/1d)Pn(1.53\times10^{-5}/1.13\times10^{-5})$$
$$(k_p)_{SHW}=0.30\ d^{-1}.$$

(iv) From this value, $k_{pE}$ was found to be 0.14 d−1 using equation 5 under paragraph (c)(2)(vii) of this section:

$$k_{pE}=0.45(0.30\ d^{-1})=0.14d^{-1}.$$

(v) From measurements in pure water, $k_D$ for chemical A was found to be 0.085 $d^{-1}$. Because the ratio of $(k_p)_{SHW}/k_D(=3.5)$ is greater than 2, Phase 3 experiments were started.

(d) *Phase 3—Indirect photoreaction with actinometer: Calculation of* $k_{IE}$ *and* $k_{pE}$—(1) *Introduction and purpose.*

(i) The purpose of Phase 3 is to measure $k_{Io}$, the indirect photolysis rate constant in tubes, and then to calculate $k_{pE}$ for the test chemical in a natural water. If the approximate $(k_p)_{SHW}$ determined in Phase 2 is not significantly greater than $k_D$ measured for the experiment date of Phase 2, then Phase 3 is unnecessary because the test chemical is not subject to indirect photoreaction.

(ii) In the case $(k_p)_{SHW}$ is significantly larger than $k_D$, Phase 3 is necessary. The rate constant $(k_p)_{SHW}$ is used to choose an actinometer composition that matches the actinometer rate to the test chemical rate. Test chemical solutions in SHW and in pure water buffer are then irradiated in sunlight in parallel with actinometer solutions, all in tubes.

(iii) The actinometer used is the *p*-nitroacetophenone-pyridine (PNAP/PYR) system developed by Dulin and Mill (1982) under paragraph (f)(5) of this section and is used in two EPA test guidelines (USEPA (1984) under paragraphs (f) (14) and (15) of this section). By varying the pyridine concentration, the PNAP photolysis half-life can be adjusted over a range of several hours to several weeks. The starting PNAP concentration is held constant.

(iv) SHW is subject to photobleaching that decreases its ability to promote indirect photolysis based on its ability to absorb sunlight. This effect will be significant when the test period exceeds a few days. To correct for photobleaching, tubes containing SHW are irradiated in action to the other tubes above.

(v) At any time, the loss of test chemical is given by Equation 8 assuming actinometric correction to constant light flux:

*Equation 8*

$$-(d[C]/dt)=k_I[C]+k_D[C].$$

(vi) The indirect photolysis rate constant, $k_I$, is actually time dependent because SHW photobleaches; the rate constant $k_I$, after pre-aging, obeys the formula:

*Equation 9*

$$k_I=k_{Io}\ \exp(-kt),$$

in which $k_{Io}$ is the initial indirect photoreaction rate constant and k is the SHW photobleaching rate constant. After substituting equation 9 for $k_I$ in Equation 8 under paragraph (d)(1)(v) of this section, and rearranging, one obtains

$$-(d[C]/[C]=k_{Io}[\exp(-kt)]dt+k_Ddt.$$

This expression is integrated to give Equation 10:

*Equation 10*

$$Pn(C_o/C)_{SHW}=(k_{Io}/k)[1-\exp(-kt)]+k_Dt.$$

The term $(k_{Io}/k)$ can now be evaluated. Since in pure water, $Pn(C_o/C)_W=k_Dt$, then subtracting this equation from Equation 10 gives

*Equation 11*

$$Pn(C_o/C)_{SHW}\text{-}Pn(c_o/C)_W=(k_{Io}/k)[1\text{-}\exp(\text{-}kt)].$$

The photobleaching fraction, [1-exp(-kt)], is equivalent to the expression $[1\text{-}(A_{370}/A^{\circ}_{370})]$, where $A^{\circ}_{370}$ and $A_{370}$ are the absorbances at 370 nm, and are proportional to humic sensitizer content at times zero and t. Therefore, $(k_{Io}/k)$ is derived from the slope of a linear regression using $[Pn(C_o/C)_{SHW}\text{-}Pn(C_o/C)_W]$ as the dependent variable and $[1\text{-}(A_{370}/A^{\circ}_{370})_{SHW}]$ as the independent variable.

(vii) To evaluate $k_{Io}$, the parameter k has to be evaluated under standard sunlight conditions. Therefore, the photolysis rate constant for the PNAP/PYR actinometer ($k_A$) is used to evaluate k by linear regression on Equation 12:

*Equation 12*

$$Pn(A^{\circ}_{370}/A_{370})=(k/k_A)Pn(C_o/C)_{PNAP},$$

where the slope is $(k/k_A)$ and the value of $k_A$ is calculated from the concentration of pyridine and the absorption of light by PNAP: $k_A=2.2(0.0169)[PYR]k_a$. Values of $k_a$ are listed in the following Table 1.

TABLE 1—DAY AVERAGED RATE CONSTANT ($k_a$) [1] FOR SUNLIGHT ABSORPTION BY PNAP AS A FUNCTION OF SEASON AND DECADIC LATITUDE [2]

| Latitude | Season | | | |
|---|---|---|---|---|
| | Spring | Summer | Fall | Winter |
| 20°N | 515 | 551 | 409 | 327 |
| 30°N | 483 | 551 | 333 | 232 |
| 40°N | 431 | 532 | 245 | 139 |
| 50°N | 362 | 496 | 154 | 64 |

[1] $k_a = @\ e_{sa} L_{\lambda}$ in the units of day $^{-1}$, (Mill et al. (1982) under paragraph (f)(10) of this section).

[2] For use in Equation 15 under paragraph (d)(2)(i) of this section.

The value of $k_{io}$ is then given by Equation 13:

*Equation 13*

$$k_{io}=(k_{io}/k)(k/k_A)k_A.$$

(viii) To obtain $k_D$, determine the ratio $(k_D/k_A)$ from a linear regression of $Pn(C_o/C)_W$ versus $Pn(C_o/C)_{PNAP}$ according to Equation 13a:

*Equation 13a*

$$Pn(C_o/C)_W=(k_D/k_A)Pn(C_o/C)_{PNAP}.$$

The slope is $(k_D/k_A)$, and $k_D$ is obtained by multiplication of this slope with the known value of $k_A$: i.e., $k_D=(k_D/k_A)k_A$.

(ix) Then, $(k_p)_{SHW}$ values in SHW are determined by summing $k_D$ and $K_{io}$ as follows:

*Equation 14*

$$(k_p)_{SHW}=k_{io}+k_D.$$

(x) Finally, $k_{pE}$ is calculated from the precise relationship, Equation 5a:

*Equation 5a*

$$k_{pE}=0.455(k_p)_{SHW}.$$

(2) *Procedure.* (i) Using the test chemical photoreaction rate constant in round tubes, $(k_p)_{SHW}$ determined in Phase 2 under paragraph (c) of this section, and the absorption rate constant, $k_a$ found in Table 1, under paragraph (d)(1)(vii) of this section, calculate the molar pyridine concentration required by the PNAP/PYR actinometer using Equation 15:

*Equation 15*

$$[PYR]/M=26.9[(k_p)_{SHW}/k_a].$$

This pyridine concentration makes the actinometer rate constant match the test chemical rate constant.

(A) The variable $k_a$ (= @ $e_{sa}$ $L_{\lambda}$) is equal to the day-averaged rate constant for sunlight absorption by PNAP (USEPA (1984) under paragraph (f)(14) of this section; Mill et al. (1982) under paragraph (f)(10) of this section, Zepp and Cline (1977) under paragraph (f)(19) of this section) which changes with season and latitude.

(B) The variable $k_a$ is selected from Table 1 under paragraph (d)(1)(vii) of this section for the season nearest the mid-experiment date of Phase 2 studies and the decadic latitude nearest the experimental site.

(ii) Once [PYR] is determined, an actinometer solution is prepared by adding 1.00 mL of 1.0 x $10^{-3}$ M (0.165 gms/100 mL) PNAP stock solution (in $CH_3$ CN solvent) and the required volume, V, of PYR to a 1 liter volumetric flask. The flask is then filled with distilled water to give 1 liter of solution. The volume V can be calculated from Equation 16:

*Equation 16*

$$V/mL=[PYR]/0.0124.$$

The PNAP/PYR solutions should be wrapped with aluminum foil and kept out of bright light after preparation.

(iii) The following solutions should be prepared and individually added in 6.00 mL aliquots to 12/100 mm quartz sample tubes; 8 tubes should be filled with each solution:

(A) PNAP/PYR actinometer solution.

(B) Test chemical in pH 7.0, 0.010 M phosphate buffer.

(C) Test chemcial in pH 7.0, 0.010 M phosphate buffer/SHW.

(D) pH 7.0, 0.010 M phosphate buffer/SHW. Four tubes of each set are wrapped in foil and used as controls.

(iv) The tubes are placed in the photolysis rack (Phase 2, Procedure) at 0900 hours on day zero, with the controls taped to the bottom of the rack. One tube of each composition is removed, along with their respective controls, according to a schedule found in Table 2, which categorizes sampling times on the basis of $(k_p)_{SHW}$ determined in Phase 1.

TABLE 2—CATEGORY AND SAMPLING PROCEDURE FOR TEST AND ACTINOMETRY SOLUTIONS

| Category | $k_p$ $(d^{-1})_{SHW}$ | Sampling procedure |
|---|---|---|
| A | 5.5 J $K_p$ J 0.69 | Sample at 0, 1, 2, 4, and 8h. |
| B | 0.69>$k_p$ J 0.017 | Sample at 0, 1, 2, 4, and 8d. |
| C | 0.17>$k_p$ J 0.043 | Sample at 0, 4, 8, 16, and 32d. |

(v) The tubes containing PNAP, test chemical, and their controls are analyzed for residual concentrations soon after the end of the experiment. PNAP is conveniently analyzed by HPLC, using a 30 cm $C_{18}$ reverse phase column and a uv detector set at 280 nm. The mobile phase is 2 percent acetic acid, 50 percent acetonitrile and 48 percent water (2 mL/min flow rate). Tubes containing only SHW (solution D) should be analyzed by absorption spectroscopy at 370 nm after storage at 4°C in the dark. The absorbance range to be measured is 0.05 to 0.01 AU (1 cm).

(vi) If controls are well-behaved and show no significant loss of chemical or absorbance change, then $k_I$ can be calculated. In tabular form (see Table 4 under paragraph (d)(6)(iii)(A) of this section) arrange the quantities $Pn(C_o/C_t)_{SHW}$, $Pn(C_o/C_t)_{SHW}$, $[1-(A_{370}/A^o_{370})]$, $Pn(A^o_{370}/A_{370})$, and $Pn(C_o/C)_{PNAP}$ in order of increasing time. According to Equation 11 under paragraph (d)(1)(vi) of this section in the form of Equation 17,

*Equation 17*

$$Pn(C_o/C)_{SHW}-Pn(C_o/C)_W=(k_{Io}/k)[1-(A_{370}/A^o_{370})],$$

plot the quantities $[Pn(C_o/C_t)_{SHW}-Pn(C_o/C_t)_W]$ versus the independent variable $[1-(A_{370}/A^o_{370})]$. Obtain the slope (S1) by least square linear regression. Under the assumptions of the protocol, $S1=(k_{Io}/k)$.

(vii) According to Equation 12 under paragraph (d)(1)(vii) of this section, plot the quantities $Pn(A^o_{370}/A_{370})$ versus the independent variable $Pn(C_o/C_t)_{PNAP}$. Obtain the slope (S2) by least squares linear regression on Equation 12 under paragraph (d)(1)(vii) of this section. Under the assumptions of the protocol, $S2=(k/k_A)$.

(viii) Then, using Equation 13a under paragraph (d)(1)(vii) of this section, determine the slope (S3) by least squares linear regression. Under the assumptions of the protocol, S3 is equal to $(k_D/k_A)$.

(ix) From Equation 18

*Equation 18*

$$k_A=0.0372[PYR]k_a,$$

calculate $k_A$ using $k_a$ values found in Table 1 under paragraph (d)(1)(vii) of this section. The value of $k_a$ chosen must correspond to the date closest to the mid-experiment date and latitude closest to that of the experimental site.

(x) The indirect photoreaction rate constant, $k_{Io}$, is determined using Equation 19,

*Equation 19*

$$k_{Io}=(S1)(k_A)(S2),$$

by incorporating the quantities $k_A$, S1, and S2 determined as described in paragraphs (d)(2) (ix), (vi), and (vii) of this section, respectively.

(xi) The rate constant $k_D$ is calculated from Equation 20,

*Equation 20*

$$k_D=(S3)(k_A),$$

using the quantities S3 and $k_A$ determined as described above.

(xii) Then, $(k_p)_{SHW}$ is obtained by summing $k_D$ and $k_{Io}$, as described by Equation 14 in paragraph (d)(1)(ix) of this section:

*Equation 14*

$$(k_p)_{SHW}=k_{Io}+k_D.$$

(xiii) Finally, $k_{pE}$ is obtained by multiplying $(k_p)_{SHW}$ by the factor 0.455, as described by Equation 5a in paragraph (d)(1)(x) of this section:

*Equation 5a*

$k_{pE}=0.455\,(k_p)_{SHW}$

As determined, $k_{pE}$ is the net environmental photoreaction rate constant. It applies to clear sky conditions and is valid for predicting surface photoreaction rates in an average humic containing freshwater body. It is strictly valid only for the experimental latitude and season.

(3) *Criteria for Phase 3.* As in Phase 2, Phase 3 tests are assumed valid if the dark controls are well behaved and show no significant loss of chemical. In such a case, loss of test chemical in irradiated samples is due to photoreaction.

(4) *Rationale.* Simultaneous irradiation of a test chemical and actinometer provide a means of evaluating sunlight intensities during the reaction period. Parallel irradiation of SHW solutions allows evaluation of the extent of photobleaching and loss of sensitizing ability of the natural water.

(5) *Scope and limitations of Phase 3 protocol.* Test chemicals that are classified as having half-lives in SHW in the range of 1 hour to 50 days in Phase 2 listing are suitable for use in Phase 3 testing. Such chemicals have photoreaction half-lives in a range accommodated by the PNAP/PYR actinometry in sunlight and also accommodate the persistence of SHW in sunlight.

(6) *Illustrative example.* (i) From Phase 2 testing, under paragraph (c)(6)(iii) of this section, chemical A was found to have a photolysis rate constant, $(k_p)_{SHW}'$ of 0.30 $d^{-1}$ in fall in round tubes at latitude 33°N. Using Table 1 under paragraph (d)(1)(vii) of this section for 30°N, the nearest decadic latitude, a fall value of $k_a$ equal to 333 $d^{-1}$ is found for PNAP. Substitution of $(k_p)_{SHW}$ and $k_a$ into Equation 15 under paragraph (d)(2)(i) of this section gives [PYR] = 0.0242 M. This is the concentration of pyridine that gives an actinometer rate constant of 0.30 $d^{-1}$ in round tubes in fall at this latitude.

(ii) The actinometer solution was made up by adding a volume of pyridine (1.95 mL) calculated from equation 16 under paragraph (d)(2)(ii) of this section to a 1 liter volumetric flask containing 1.00 mL of $1.00 \times 10^{-3}$ M PNAP in acetonitrile. The flask was filled to the mark with distilled water to give final concentrations of [PYR]=0.0242 M and [PNAP]=$1.00\times10^{-6}$ M. Ten tubes of each of the following solutions were placed in the photolysis rack at 1,200 hours on day zero:

(A) Chemical A ($1.53\times10^{-5}$M) in standard SHW (0.010 M, pH 7 phosphate buffer).

(B) Chemical A ($1.53\times10^{-5}$), in 0.010 M, pH 7 phosphate buffer.

(C) SHW standard solution diluted with water 0.90 to 1.00 to match solution A.

(D) PNAP/PYR actinometer solution. Ten additional foil-wrapped controls of each mixture were taped to the bottom of the rack.

(iii) The test chemical had been placed in category B, Table 2 under the paragraph (d)(2)(iv) of this section, on the basis of its Phase 2 rate constant under paragraph (c) of this section. Accordingly, two tubes of each irradiated solution and two tubes of each blank solution were removed at 0, 1, 2, 4, and 8 days at 1,200 hours. The averaged analytical results obtained at the end of the experiment are shown in the following Table 3.

TABLE 3—CHEMICAL ANALYTICAL RESULTS FOR ILLUSTRATIVE EXAMPLE, PHASE 3

| Day | $10^5[C]^{SHW}$, M | $10^5[C]^{W}$, M | $A^{SHW}_{370}$ | $10^6$ [PNAP], M |
|---|---|---|---|---|
| 0 | 1.53 | 1.53 | 0.0500 | 1.00 |
| 1 | 1.03 | 1.40 | 0.0470 | 0.810 |
| 2 | 0.760 | 1.30 | 0.0440 | 0.690 |
| 4 | 0.300 | 1.01 | 0.0370 | 0.360 |
| 8 | 0.130 | 0.800 | 0.0320 | 0.220 |

Data for solutions A through D are given in column 2 through 5, respectively. No significant chemical loss was found in the dark controls.

(A) From these items the functions Pn$(C_o/C)_{SNW}$, Pn$(C_o/C)_W$, $[1-(A_{370}/A°_{370})_{SNW}]$, Pn$(A°_{370}/A_{370})$, and Pn$(C_o/C)_{PNAP}$ were calculated, as shown in the following Table 4 which was derived from Table 3 under paragraph (d)(6)(iii) of this section:

TABLE 4—PHOTOREACTION FUNCTION FOR ILLUSTRATIVE EXAMPLES, PHASE 3, DERIVED FROM TABLE 3

| Day | Pn$(C_o/C)_{SNW}$ | Pn$(C_o/C)_W$ | $1-(A_{370}/A°_{370})$ | Pn$(A°_{370}/A_{370})$ | Pn$(C_o/C)_{PNAP}$ |
|---|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 | 0 |
| 1 | 0.396 | 0.0888 | 0.0600 | 0.0618 | 0.211 |
| 2 | 0.700 | 0.163 | 0.120 | 0.128 | 0.371 |
| 4 | 1.629 | 0.415 | 0.260 | 0.301 | 0.968 |
| 8 | 2.465 | 0.648 | 0.360 | 0.446 | 1.514 |

(B) Slope $S1=(k_{io}/k)$ was calculated according to Equation 17 under paragraph (d)(2)(vi) of this section and was found to be 4.96 by a least squares regression with a correlation coefficient equal to 0.9980. The following Figure 1 shows a plot of Equation 17 under paragraph (d)(2)(vi) of this section and its best-fit line.

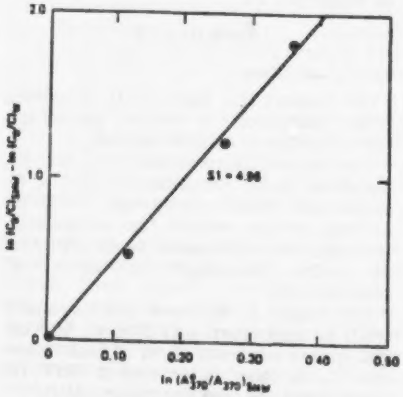

Figure 1—Graphic determination of $S1=(k_{io}/k)$ based on Equation 17 under paragraph (d)(2)(vi) of this section.

(C) Slope $S2=(k/k_A)$ was also derived from Table 4 under paragraph (d)(6)(iii)(A) of this section by a fit of Pn$(A°_{370}/A_{370})_{SNW}$ and Pn$(C_o/C)_{PNAP}$ to Equation 12 under paragraph (d)(1)(vii) of this section. This plot is displayed in the following Figure 2; the slope S2 was found to be 0.295 and the correlation coefficient was equal to 0.9986.

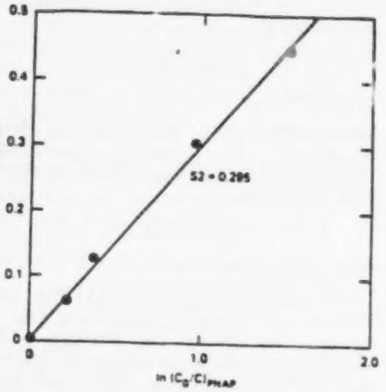

Figure 2—Graphic determination of $S2=(k/k_A)$ based on Equation 12 under paragraph (d)(1)(vii) of this section.

(D) Using the data in columns 3 and 6 in Table 4 under paragraph (d)(6)(iii)(A) of this section, slope S3 was calculated by regression from Equation 13a under paragraph (d)(1)(viii) of this section and was found to be 0.428 with correlation coefficient euqal to 0.99997.

(E) Using Equation 18 under paragraph (d)(2)(ix) of this section, $k_A$ was found to be $=0.300\ d^{-1}$.

(F) The values of S1, S2, and $k_A$ were then combined in Equation 19 under paragraph (d)(2)(x) of this section to give $k_{io}$ as follows:

*Equation 19*

$$k_{io}=(4.96)(0.300)(0.295)=0.439\ d^{-1}.$$

(G) The rate constant $k_D$ was calculated from the product of S3 and $k_A$ as expressed in Equation 20 under paragraph (d)(2)(xi) of this section as follows:

*Equation 20*

$$k_D=(0.428)(0.300)=0.128d^{-1}.$$

(H) The sum of $k_D$ and $k_{io}$ was multiplied by 0.455 to obtain $k_{pE}$ as follows:

*Equation 21*

$$k_{pE}=(0.455)(0.439+0.128)d^{-1}=0.258\ d^{-1}.$$

(I) Since $k_{pE}$ is a first-order rate constant, the half-life, $t_{1/2E}$, is given by Equation 22:

*Equation 22*

$$t_{1/2E}=0.693/k_{pE}.$$

Substituting the value of $k_{pE}$ from Equation 21 under paragraph (d)(6)(iii)(H) of this section in Equation 22 yielded

*Equation 23*

$$t_{1/2E}=0.693/0.258d^{-1}=2.7\ d.$$

(e) *Data and reporting*—(1) *Test conditions*—(i) *Specific analytical and recovery procedures.* (A) Provide a detailed description or reference for the analytical procedures used, including the calibration data and precision.

(B) If extraction methods were used to separate the solute from the aqueous solution, provide a description of the extraction method as well as the recovery data.

(ii) *Other test conditions.* (A) Report the site and latitude where the photolysis experiments were carried out.

(B) Report the dates of photolysis, weather conditions, times of exposure, and the duration of exposure.

(C) If acetonitrile was used to solubilize the test chemical, report the volume percent.

(D) If a significant loss of test chemical occurred in the control solutions for pure water and SHW, indicate the causes and how they were eliminated or minimized.

(2) *Test data report*—(i) Phase 2 Screening Test under paragraph (c) of this section. (A) Report the initial molar concentration of test chemical, $C_o$, in pure water and SHW for each replicate and the mean value.

(B) Report the molar concentration of test chemical, $C_t$, in pure water and SHW for each replicate and the mean value for each time point t.

(C) Report the molar concentration of test chemical for each replicate control sample and the mean value for each time point.

(D) Report the values of $(k_p)_{SHW}$ and $(k_p)_W$ for the time point t in which the fraction of test chemical photoreacted is in the range 20 to 80 percent.

(E) If small losses of test chemical were observed in SHW and pure water, report a first-order rate constant loss, $(k_p)_{loss}$. Calculate and report $(k_p)_{obs}$ for SHW and/or pure water. Calculate and report the corrected first-order rate constant for SHW and/or pure water using the relationship expressed in Equation 24:

*Equation 24*

$$k_p=(k_p)_{obs}-(k_p)_{loss}.$$

(F) Report the value of R calculated from Equation 4 under paragraph (c)(2)(vi)(D)(*4*) of this section.

(G) Report the values of $k_{pE}$ and $k_{DE}$ obtained from Equations 5 and 6, respectively under paragraph (c)(2)(vii) of this section; report the corresponding half-life calculated from Equation 22 under paragraph (d)(6)(iii)(I) of this section.

(ii) Phase 3—Indirect photoreaction with actinometer. (A) Report the initial molar concentration of test chemical, $C_o$, in pure water and in SHW for each replicate and the mean value.

(B) Report the initial absorbance $A^o_{370}$ of the SNW solution.

(C) Report the initial molar concentration of PNAP of each replicate and the mean value in the actinometer. Report the concentration of pyridine used in the actinometer which was obtained from Equation 15 under paragraph (d)(2)(i) of this section.

(D) Report the time and date the photolysis experiments were started, the time and date the experiments were completed, and the elapsed photolysis time in days.

(E) For each time point t, report the separate values of the absorbance of the SHW solution, and the mean values.

(F) For each time point for the controls, report the separate values of the molar concentrations of test chemical in pure water and SHW, and the absorbance of the SHW solution, and the mean values.

(G) Tabulate and report the following data: t, $[C]^{SHW}$, $[C]^{W}$, $A^{SNW}_{370}$, [PNAP].

(H) From the data in (G), tabulate and report the following data: t, $Pn(C_o/C)_{SNW}$, $Pn(C_o/C)_W$, $[1-(A_{370}/A^o_{370})SNW]$, $Pn(A^o_{370}/A_{370})$, $Pn(C_o/C)_{PNAP}$.

(I) From the linear regression analysis of the appropriate data in step (H) in Equation 17 under paragraph (d)(2)(vi) of this section, report the slope S1 and the correlation coefficient.

(J) From the linear regression analysis of the appropriate data in step (H) in Equation 12 under paragraph (d)(1)(vii) of this section, report the slope S2 and the correlation coefficient.

(K) From the linear regression analysis of the appropriate data in step (H) in Equation 13a under paragraph (d)(1)(viii) of this section, report the slope S3 and the correlation coefficient.

(L) If loss of chemical was observed during photolysis in pure water and SHW, then report the data $Pn(C_o/C)_{corr}$, $Pn(C_o/C)_{obs}$, $Pn(C_o/C)_{loss}$ as described in paragraph (e)(2)(E) of this section. Repeat steps (H), (I), (J), (K) where applicable and report S1, S2, S3 and the corresponding correlation coefficients.

(M) Report the value of the actinometer rate constant obtained from Equation 18 under paragraph (d)(2)(ix) of this section.

(N) Report the value of $k_{io}$ obtained from Equation 19 under paragraph (d)(2)(x) of this section.

(O) Report the value of $k_D$ obtained from Equation 20 under paragraph (d)(2)(xi) of this section.

(P) Report the value of $(k_{pE})_{SHW}$, obtained from Equation 14 under paragraph (d)(1)(ix) of this section, and the value of $k_{pE}$ obtained from Equation 5a under paragraph (d)(1)(x) of this section.

(Q) Report the half-life, $t_{1/2E}$, obtained from Equation 22 under paragraph (d)(6)(iii)(I) of this section.

(f) *References.* For additional background information on this test guideline the following references should be consulted.

(1) Cooper W.J., Zika R.G. "Photochemical formation of hydrogen peroxide in surface and ground waters exposed to sunlight." *Science*, 220:711. (1983).

(2) Draper W.M., Crosby D.G. "The photochemical generation of hydrogen peroxide in natural waters." *Archives of Environmental Contamination and Toxicology*, 12:121. (1983).

(3) Draper, W.M. and Crosby D.G. "Solar photooxidation of pesticides in dilute hydrogen peroxide." *Journal of Agricultural and Food Chemistry*, 32:231. (1984).

(4) Draper W.M., Crosby D.G. "Hydrogen peroxide and hydroxyl radical: Intermediates in indirect photolysis reactions in water." *Journal of Agricultural and Food Chemistry*, 29:699. (1981).

(5) Dulin D., Mill T. "Development and evaluation of sunlight actinometers." *Environmental Science and Technology*, 6:815. (1982).

(6) Haag H.R., Hoigne J., Gassman E., Braun A.M. "Singlet oxygen in surface waters—Part I; Furfuryl alcohol as a trapping agent." *Chemosphere*, 13:631. (1984).

(7) Haag W.R., Hoigne J., Gassman E., Braun A.M. "Singlet oxygen in surface waters—Part II: Quantum yields of its production by some natural humic materials as a function of wavelength." *Chemosphere*, 13:641. (1984).

(8) Mill T., Winterle J.S., Fischer A., Tse D., Mabey W.R., Drossman H., Liu A., Davenport J.E. Toxic substances process data generation and protocol development. Work assignment 12, test standard development. "Section 3. Indirect photolysis." Draft final report. EPA Contract No. 68-03-2981. Environmental Research Laboratory, Office of Research and Development,

EPA, Athens, GA, and Office of Toxic Substances, EPA, Washington, DC. (1984).

(9) Mill T., Mabey W.R., Bomberger D.C., Chou T.W., Hendry D.G., Smith J.H. "Laboratory protocols for evaluating the fate of organic chemicals in air and water. Chapter 3. Photolysis in water. Chapter 4. Oxidation in water." EPA 600/3-82-022. Environmental Research Laboratory, Office of Research and Development, EPA, Athens, GA. (1981).

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(11) Mill T., Davenport J.E., Winterle J.S., Mabey W.R., Dossman H., Tse D., Liu A. Toxic substances process data generation and protocol development. Work assignment 12. "Appendix B. Upper-tier protocol for direct photolysis in water." Draft final report. EPA Contract No. 68-03-2981. Environmental Research Laboratory, Office of Research and Development, EPA, Athens, GA, and Office of Toxic Substances, EPA, Washington, DC. (July 1983).

(12) Winterle J.S., Mill T. Toxic substances process data generation and protocol development. Work assignment 18. "Indirect photoreaction protocol." Draft EPA special report. EPA Contract No. 68-03-2981. Environmental Research Laboratory, Office of Research and Development, EPA, Athens, GA and Office of Toxic Substances, EPA, Washington, DC. (1985).

(13) Mill T., Hendry D.G., Richardson H. "Free radical oxidants in natural waters." *Science*, 207:886. (1980).

(14) U.S. Environmental Protection Agency (USEPA), Office of Toxic Substances (OTS). "Chemical fate test guidelines. Test guideline (CG, CS-6000). Photolysis in aqueous solution." EPA-560/6-84-003. NTIS publication PB-84-233287. (1984).

(15) USEPA, OTS. "Chemical fate test guidelines. Test guildeline (CG, CS-6010). Laboratory determination of the direct photolysis reaction quantum yield in aqueous solution and sunlight photolysis." EPA-560/6-84-003. NTIS publication PB-84-233287. (1984).

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(19) Zepp R.G., Cline D.M. "Rates of direct photolysis in aquatic environments." *Environmental Science and Technology*, 11:359. (1977).

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[53 FR 34522, Sept. 7, 1988; 53 FR 37393, Sept. 26, 1988]

## Subpart C—Provisional Environmental Effects Guidelines

### § 795.120 Gammarid acute toxicity test.

(a) *Purpose*. This guideline is intended for use in developing data on the acute toxicity of chemical substances and mixtures subject to environmental effects test regulations under the Toxic Substances Control Act (TSCA) (Pub. L. 94-469, 90 Stat. 2003 (15 U.S.C. 2601 *et. seq.*)). This guideline describes a test to develop data on the acute toxicity of chemicals to gammarids. The United States Environmental Protection Agency (EPA) will use data from this test in assessing the hazard of a chemical to aquatic organisms.

(b) *Definitions.* The definitions in section 3 of TSCA and in Part 792 of this chapter, Good Laboratory Practice Standards, apply to this test guideline. The following definitions also apply to this guideline:

"Death" means the lack of reaction of a test organism to gentle prodding.

"Flow-through" means a continuous or an intermittent passage of test solution or dilution water through a test chamber or a holding or acclimation tank, with no recycling.

"LC50" means the median lethal concentration, i.e., that concentration of a chemical in air or water killing 50 percent of the test batch of organisms within a particular period of exposure (which shall be stated).

"Loading" means the ratio of the biomass of gammarids (grams, wet weight) to the volume (liters) of test solution in either a test chamber or passing through it in a 24-hour period.

"Solvent" means a substance (e.g., acetone) which is combined with the test substance to facilitate introduction of the test substance into the dilution water.

"Static system" means a test chamber in which the test solution is not renewed during the period of the test.

(c) *Test procedures*—(1) *Summary of the test.* In preparation for the test, test chambers are filled with appropriate volumes of dilution water. If a flow-through test is performed, the flow of dilution water through each chamber is adjusted to the rate desired. In a static test, the test substance is introduced into each test chamber. In a flow-through test, the rate in which the test substance is added is adjusted to establish and maintain the desired concentration of test substance in each test chamber. The test is started by randomly introducing gammarids, which have been acclimated to the test conditions, into the test chambers. Gammarids in the test chambers are observed periodically during the test; the dead gammarids are removed and the findings recorded. Dissolved oxygen concentration, pH, temperature, and the concentration of test substance in test chambers are measured at specified intervals. Data collected during the test are used to develop concentration—response curves and LC50 values for the test substance.

(2) [Reserved]

(3) *Range-finding test.* (i) A range-finding test should be conducted to establish test substance concentrations to be used for the definitive test.

(ii) The gammarids shall be exposed to a wide-range of concentrations of the test substance (e.g., 1, 10, 100 mg/l, etc.), usually under static conditions.

(iii) A minimum of five gammarids should be exposed to each concentration of test substance for a period of 96 hours. The exposure period may be shortened if data suitable for determining concentrations in the definitive test can be obtained in less time. Nominal concentrations of the test substance may be acceptable.

(4) *Definitive test.* (i) The purpose of the definitive test is to determine the 24, 48, 72, and 96—hour LC50 values and the concentration-response curves.

(ii) A minimum of 20 gammarids per concentration shall be exposed to five or more concentrations of the test substance chosen in a geometric series in which the ratio is between 1.5 and 2.0 (e.g., 2, 4, 8, 16, 32, 64 mg/L). The range and number of concentrations to which the organisms are exposed shall be such that in 96 hours there is at least one concentration resulting in mortality greater than 50 and less than 100 percent, and one concentration causing greater than zero and less than 50 percent mortality. An equal number of gammarids may be placed in two or more replicate test chambers. Solvents should be avoided, if possible. If solvents have to be used, a solvent control, as well as a dilution control, shall be tested at the highest solvent concentration employed in the treatments. The solvent should not be toxic or have an effect on the toxicity of the test substance. The concentration of solvent should not exceed 0.1 ml/L.

(iii) Every test shall include a concurrent control using gammarids from the same population or culture container. The control group shall be exposed to the same dilution water, conditions and procedures, except that none of the test substance shall be is added to the chamber.

(iv) The dissolved oxygen concentration, temperature and pH of the test solution shall be measured at the beginning of the test and at 24, 48, 72 and 96 hours in at least one replicate each of the control, and the highest, lowest and middle test concentrations.

(v) The test duration is 96 hours. The test is unacceptable if more than 10 percent of the control organisms die during the test.

(vi) In addition to death, any abnormal behavior or appearance shall also be reported.

(vii) Gammarids shall be randomly assigned to the test chambers. Test chambers shall be positioned within the testing area in a random manner or in a way in which appropriate statistical analyses can be used to determine whether there is any variation due to placement.

(viii) Gammarids shall be introduced into the test chambers after the test substance has been added.

(ix) Observations on compound solubility shall be recorded. The investigator should record the appearance of surface slicks, precipitates, or material adhering to the sides of the test chambers.

(5) [Reserved]

(6) *Analytical measurements*—(i) *Water quality analysis.* The hardness, acidity, alkalinity, pH, conductivity, TOC or COD, and particulate matter of the dilution water shall be measured at the beginning of each definitive test.

(ii) *Collection of samples for measurement of test substance.* Each sample to be analyzed for the test substance concentrations shall be taken at a location midway between the top, bottom, and sides of the test chamber. Samples should not include any surface scum or material dislodged from the bottom or sides. Samples shall be analyzed immediately or handled and stored in a manner which minimizes loss of test substance through microbial degradation, photogradation, chemical reaction, volatilization, or sorption.

(iii) *Measurement of test substance.* (A) For static tests, the concentration of dissolved test substance (that which passes through a 0.45 micron filter) shall be measured in each test chamber at least at the beginning (zero-hour, before gammarids are added) and at the end of the test. During flow-through tests, the concentration of dissolved test substance shall be measured in each test chamber at least at 0 and 96-hours and in at least one chamber whenever a malfunction of the test substance delivery system is observed.

(B) The analytical methods used to measure the amount of test substance in a sample shall be validated before beginning the test. This involves adding a known amount of the test substance to each of three water samples taken from a chamber containing dilution water and the same number of gammarids as are placed in each test chamber. The nominal concentrations of the test substance in these samples should span the concentration range to be used in the test. Validation of the analytical method should be performed on at least two separate days prior to starting the test.

(C) An analytical method is not acceptable if likely degradation products of the test substance give positive or negative interferences, unless it is shown that such degradation products are not present in the test chambers during the test.

(D) Among replicate test chambers, the measured concentrations shall not vary more than 20 percent. The measured concentration of the test substance in any chamber during the test shall not vary more than plus or minus 30 percent from the measured concentration in that chamber at zero time.

(E) The mean measured concentration of dissolved test substance shall be used to calculate all LC50's and to plot all concentration-response curves.

(d) *Test conditions for definitive test*—(1) *Test species*—(i) *Selection.* (A) The amphipods, *Gammarus fasciatus*, *G. pseudolimnaeus*, and *G. lacustris* are specified for this test.

(B) Gammarids can be cultured in the laboratory or collected from natural sources. If collected, they must be held in the laboratory for at least 14 days prior to testing.

(C) Gammarids used in a particular test shall be of similar age and/or size and from the same source or culture population.

(ii) *Acclimation.* If the holding water is from the same source as the dilution water, acclimation to the dilution water shall be done gradually over a 48-hour period. The gammarids then shall be held at least 7 days in the dilution water prior to testing. Any changes in water temperature should not exceed 2 °C per day. Gammarids should be held for a minimum of 7 days at the test temperature prior to testing.

(iii) *Care and handling.* Gammarids shall be cultured in dilution water under similar environmental conditions to those used in the test. Organisms shall be handled as little as possible. When handling is necessary it should be done as gently, carefully and quickly as possible. During culturing and acclimation, gammarids shall be observed carefully for signs of stress and mortality. Dead and abnormal individuals shall be discarded.

(iv) *Feeding.* The organisms shall not be fed during testing. During culturing, holding, and acclimation, a sufficient quantity of deciduous leaves, such as maple, aspen, or birch, should be placed in the culture and holding containers to cover the bottom with several layers. These leaves should be aged for at least 30 days in a flow-through system before putting them in aquaria. As these leaves are eaten, more aged leaves should be added. Pelleted fish food may also be added.

(2) *Facilities*—(i) *Apparatus*—(A) Facilities needed to perform this test include:

(*1*) Containers for culturing, acclimating and testing gammarids;

(*2*) Containers for aging leaves under flow-through conditions;

(*3*) A mechanism for controlling and maintaining the water temperature during the culturing, acclimation and test periods;

(*4*) Apparatus for straining particulate matter, removing gas bubbles, or aerating the dilution water, as necessary; and

(*5*) An apparatus for providing a 16-hour light and 8-hour dark photoperiod with a 15- to 30-minute transition period.

(B) Facilities should be well ventilated and free of fumes and disturbances that may affect the test organism.

(C) Test chambers shall be covered loosely to reduce the loss of test solution or dilution water due to evaporation and to minimize the entry of dust or other particulates into the solutions.

(ii) *Construction materials.* Construction materials and equipment that may contact the stock solution, test solution or dilution water should not contain substances that can be leached or dissolved into aqueous solutions in quantities that can alter the test results. Materials and equipment that contact stock or test solutions should be chosen to minimize sorption of test substances. Glass, stainless steel, and perfluorocarbon plastic should be used wherever possible. Concrete, fiberglass, or plastic (e.g., PVC) may be used for holding tanks, acclimation tanks, and water supply systems, but they should be aged prior to use. Rubber, coopper, brass, galvanized metal, and lead should not come in contact with the dilution water, stock solution, or test solution.

(iii) *Test substance delivery system.* In flow-through tests, diluters, metering pump systems or other suitable devices shall be used to deliver the test substance to the test chambers. The system used shall be calibrated before each test. The general operation of the test substance delivery system shall be checked twice daily during a test. The 24-hour flow shall be equal to at least five times the volume of the test chamber. During a test, the flow rates should not vary more than 10 percent from one test chamber to another.

(iv) *Test chambers.* Test chambers shall contain at least one liter of test solution. Test chambers made of stainless steel should be welded, not soldered. Test chambers made of glass should be glued using clear silicone adhesive. As little adhesive as possible should be left exposed in the interior of the chamber. A substrate, such as a bent piece of stainless steel screen, should be placed on the bottom of each test chamber to provide cover for the gammarids.

(v) *Cleaning of test system.* Test substance delivery systems and test chambers should be cleaned before each test. They should be washed with detergent and then rinsed sequentially with clean water, pesticide-free acetone, clean water, and 5-percent nitric acid, followed by two or more changes of dilution water.

(vi) *Dilution water.* (A) Clean surface or ground water, reconstituted water, or dechlorinated tap water is acceptable as dilution water if gammarids will survive in it for the duration of the culturing, acclimating, and testing periods without showing signs of strees. The quality of the dilution water should be constant enough that the month-to-month variation in hardness, acidity, alkalinity, conductivity, TOC or COD, and particulate matter is not more than 10 percent. The pH should be constant within 0.4 unit. In addition, the dilution water should meet the following specifications measured at least twice a year:

| Substance | Maximum concentration |
|---|---|
| Particulate matter | 20 mg/L |
| Total organic carbon (TOC) or | 2 mg/L |
| chemical oxygen demand (COD) | 5 mg/L |
| Boron, fluoride | 100 ug/L |
| Un-ionized ammonia | 1 ug/L |
| Aluminum, arsenic, chromium, cobalt, copper, iron, lead, nickel, zinc | 1 ug/L |
| Residual chlorine | 3 ug/L |
| Cadmium, mercury, silver | 100 ng/L |
| Total organophosphorus pesticides | 50 ng/L |
| Total organochlorine pesticides plus polychlorinated biphenyls (PCBs) or | 50 ng/L |
| organic chlorine | 25 ng/L |

(B) If the dilution water is from a ground or surface water source, conductivity and total organic carbon (TOC) or chemical oxygen demand (COD) shall be measured. Reconstituted water can be made by adding specific amounts of reagent-grade chemicals to deionized or distilled water. Glass-distilled or carbon-filtered deionized water with a conductivity less than 1 micromho/cm is acceptable as the diluent for making reconstituted water.

(C) The concentration of dissolved oxygen in the dilution water shall be between 90 and 100 percent saturation. If necessary, the dilution water can be aerated before the addition of the test substance. All reconstituted water should be aerated before use.

(3) *Test parameters.* Environmental parameters during the test shall be maintained as specified below:

(i) Water temperature of 18 ± 1°C.

(ii) Dissolved oxygen concentration between 60 and 105 percent saturation.

(iii) The number of gammarids placed in a test chamber shall not be so great as to affect the results of the test. Ten gammarids per liter is the recommended level of loading for the static test. Loading requirements for the flow-through test will vary depending on the flow rate of dilution water. The loading should not cause the dissolved oxygen concentration to fall below the recommended levels.

(iv) Photoperiod of 16 hours light and 8 hours darkness.

(e) *Reporting.* The sponsor shall submit to the EPA all data developed by the test that are suggestive or predictive of toxicity. In addition, the test report shall include, but not necessarily be limited to, the following information:

(1) Name and address of the facility performing the study and the dates on which the study was initiated and completed.

(2) Objectives and procedures stated in the approved protocol, including any changes in the original protocol.

(3) Statistical methods employed for analyzing the data.

(4) The test substance identified by name, Chemical Abstracts (CAS) number or code number, source, lot or batch number, strength, purity, and composition, or other appropriate characteristics.

(5) Stability of the test substance under the conditions of the test.

(6) A description of the methods used, including:

(i) The source of the dilution water, its chemical characteristics (e.g., hardness, pH, etc.) and a description of any pretreatment.

(ii) A description of the test substance delivery system, test chambers, the depth and volume of solution in the chamber, the way the test was begun (e.g., test substance addition), the loading, the lighting, and the flow rate.

(iii) Frequency and methods of measurements and observations.

(7) The scientific name, weight, length, source, and history of the organisms used, and the acclimation procedures and food used.

(8) The concentrations tested, the number of gammarids and replicates per test concentration. The reported results should include:

(i) The results of dissolved oxygen, pH and temperature measurements.

(ii) If solvents are used, the name and source of the solvent, the nominal concentration of the test substance in the stock solution, the highest solvent concentration in the test solution and a description of the solubility determination in water and solvents.

(iii) The measured concentration of the test substance in each test chamber just before the start of the test and at all subsequent sampling periods.

(iv) In each test chamber at each observation period, the number of dead and live test organisms, the percentage of organisms that died, and the number of test organisms that showed any abnormal effects in each test chamber at each observation period.

(v) The 48, 72 and 96-hour LC50's and their 95 percent confidence limits. When sufficient data have been generated, the 24-hour LC50 value also. These calculations should be made using the mean measured test substance concentrations.

(vi) The observed no-effect concentration (the highest concentration tested at which there were no mortalities or abnormal behavioral or physiological effects), if any.

(vii) Methods and data for all chemical analyses of water quality and test substance concentrations, including method validations and reagent blanks.

(9) A description of all circumstances that may have affected the quality or integrity of the data.

(10) The names of the sponsor, study director, principal investigator, names of other scientists or professionals, and the names of all supervisory personnel involved in the study.

(11) A description of the transformations, calculations, or operations performed on the data, a summary and analysis of the data, and a statement of the conclusions drawn from the analysis. Results of the analysis of data should include the calculated LC50 value, 95 percent confidence limits, slope of the transformed concentration-response line, and the results of a goodness-of-fit test (e.g., chi-square test).

(12) The signed and dated reports prepared by any individual scientist or other professional involved in the study, including each person who, at the request or direction of the testing facility or sponsor, conducted an analysis or evaluation of data or specimens from the study after data generation was completed.

(13) The locations where all specimens, raw data, and the final report are stored.

(14) The statement prepared and signed by the quality assurance unit.

[52 FR 24462, July 1, 1987]

## Subpart D—Provisional Health Effects Guidelines

### § 795.223 Pharmacokinetic test.

(a) *Purpose.* The purpose of these tests is to determine:

(1) The bioavailability of a test substance after dermal administration.

(2) Whether or not the biotransformation of the test substance is qualitatively and quantitatively the same after dermal and oral administration.

(3) Whether or not the biotransformation of the test substance is changed qualitatively or quantitatively by repeated dosing.

(b) *Definitions*—(1) Bioavailability refers to the rate and extent to which the administered compound is absorbed, i.e., reaches the systemic circulation.

(2) Relative percent of percutaneous absorption is defined as 100 times the ratio between total urinary excretion of compound following topical administration and total urinary excretion of compound following intravenous injection.

(c) *Test procedures*—(1) *Animal selection*—(i) *Species.* The species utilized for investigating the test substance shall be the rat, a species for which historical data on the toxicity and carcinogenicity of several compounds are available and which is used extensively in percutaneous absorption studies.

(ii) *Animals.* Adult female Fischer 344 rats shall be used. The rats shall be 7 to 9 weeks old and weigh 125 to 175 grams. Prior to testing the animals shall be selected at random for each group. Animals showing signs of ill health shall not be used.

(iii) *Animal care.* (A) The animals should be housed in environmentally controlled rooms with 10 to 15 air changes per hour. The rooms should be maintained at a temperature of 25±2° C and humidity of 50±10 percent with a 12-hour light/dark cycle per day. The rats should be kept in a quarantine facility for at least 7 days prior to use.

(B) During the acclimatization period, the rats should be housed in cages on hardwood chip bedding. All animals shall be provided with conventional laboratory diets and water *ad libitum.*

(2) *Administration of test substance*—(i) *Test compound.* Test studies require the use of both nonradioactive test substance and $^{14}$C-labeled test substance. Both preparations are needed to investigate under paragraph (a)(2) of this section. The use $^{14}$C-test substance is required to investigate under paragraphs (a)(1), (2), and (3) of this section because it will facilitate the work, improve the reliability of quantitative determinations, and increase the probability of observing the presence of previously unidentified metabolities.

(ii) *Dosage and treatment.* (A) Two doses shall be used in the study, a "low" dose and a "high" dose. When administered orally, the "high" dose level should ideally induce some overt toxicity such as weight loss. The "low" dose level should correspond to a no-effect level.

(B) The same "high" and "low" doses shall be administered orally and dermally.

(C) Oral dosing shall be performed by gavage or by administering encapsulated test substance.

(D) For dermal treatment, the doses shall be applied at a volume adequate to deliver the prescribed doses. The backs of the rats should be lightly shaved with an electric clipper shortly before treatment. The dose shall be applied with a micropipette on 2 cm$^2$ of the freshly shaven skin. The dosed areas shall be occluded with an aluminum foil patch which is secured in place with adhesive tape.

(iii) *Bioavailability study in rats.* At least eight rats shall receive a single intravenous (low) dose of $^{14}$C-test substance and serial samples of blood removed from four animals at 15 minutes, 30 minutes, 1 hour, 8 hours, 24 hours, 48 hours, and 96 hours. All animals shall be housed in metabolism cages and urine and feces collected at 8, 24, 48, 72, and 96 hours. The procedure shall be repeated with eight rats in which $^{14}$C-test substance is maintained in contact with the skin for the duration of the study (96 hours). If dermal absorption cannot be demonstrated, the study should be repeated using a higher dose. Total radioactivity shall be measured in the blood, urine, and feces samples collected from all animals. The results shall be used to construct a blood concentration-time curve and to calculate bioavailability by the ratio of the total 96-hour urinary excretion of radioactivity after dermal and intravenous administration. Bioavailability is expressed as (percent dose dermal/percent dose intravenous)×100=percent dermal absorption. Urine shall be saved for metabolite identification, if it becomes necessary.

(iv) *Biotransformation in rats after oral and dermal administration.* Eight rats shall be dosed orally, and eight rats shall be dosed dermally (96-hour contact) with the high dose of $^{14}$C-test substance. The results of the bioavailability study (see paragraph (c)(2)(iii) of this section) shall be evaluated first to ensure that the dermal dose applied will result in the appearance of radioactivity in the urine. All animals shall be housed in metabolism cages allowing for separate collection of urine and feces at 8, 24, 48, 72, and 96 hours. The parent compound and any metabolite that comprises greater than 10 percent of the dose shall be identified in the urine. These results shall be qualitatively compared to the urinary excretion data obtained in the low dose bioavailability study (see paragraph (c)(2)(iii) of this section); metabolites in the low dose urine shall also be identified if a different pattern of metabolism is evident.

(v) *Repeated dosing study.* Four rats shall receive a series of single daily oral doses of nonradioactive test substance over a period of at least 14 days, followed at 24 hours after the last dose by a single oral dose of $^{14}$C-test substance. Each dose shall be at the low-dose level. If the pattern of urinary metabolite excretion is qualitatively different from that obtained with the orally dosed animals in the single-dose biotransformation study at 24 and 48 hours (see paragraph (c)(2)(iv) of this section), metabolites shall be identified in accordance with the procedure given in paragraph (c)(2)(iii) of this section.

(vi) *Skin washing study.* If greater than 10 percent of test substance is absorbed through the skin (see paragraphs (c)(2) (ii) and (iii) of this section) then a washing efficacy experiment shall be performed to assess the extent of removal of the applied test substance by washing with soap and water. Four rats should be lightly anesthetized and treated with a dermal dose of test compound previously shown to result in measurable percutaneous absorption greater than 10 percent. Soon after application (5 to 10 minutes) the treated animals shall be washed with soap and water, then housed in individual metabolism cages for excreta collection. Measurements of total radioactivity in urine and feces shall be made in the same manner as described in paragraph (c)(2)(iii) of this section.

(d) *Data and Reporting*—(1) *Treatment of results.* Data shall be summarized in tabular form.

(2) *Evaluation of results.* All observed results, quantitative or incidental, shall be evaluated by an appropriate statistical method.

(3) *Test report.* In addition to the reporting requirements as specified in the TSCA Good Laboratory Practice Standards, 40 CFR Part 792, Subpart J, the following specific information shall be reported:

(i) Species, strain, and supplier of laboratory animals.

(ii) Information on the degree (i.e., specific activity for a radiolabel) and site(s) of labeling of the test substances.

(iii) A full description of the sensitivity and precision of all procedures used to produce the data.

(iv) Relative percent absorption by the dermal route for rats administered low and high doses of $^{14}$C-test substance, compared with 100 percent of the intravenous dose.

(v) Quantity of isotope, together with percent recovery of the administered dose, in feces, urine, and blood.

(vi) Biotransformation pathways and quantities of the test substance and metabolites in urine collected after administering single high and low oral and dermal doses.

(vii) Biotransformation pathways and quantities of test substance and metabolites in urine collected after administering repeated low doses of test substance to rats.

[51 FR 40327, Nov. 6, 1986, as amended at 52 FR 24157, June 29, 1987]

## § 795.225 Dermal pharmacokinetics of DGBE and DGBA.

(a) *Purpose.* The purpose of these studies is to determine:

(1) The absorption of diethylene glycol butyl ether (DGBE) after administration by the dermal route.

(2) The biotransformation of DGBE administered dermally.

(3) The dermal absorption of DGBE and diethylene glycol butyl ether acetate (DGBA).

(b) *Test procedures*—(1) *Animal selection*—(i) *Species.* The species utilized for investigating DGBE and DGBA shall be the rat, a species for which historical data on the toxicity and carcinogenicity of many compounds are available and which is used extensively in percutaneous absorption studies.

(ii) *Animals.* Adult female Sprague Dawley rats shall be used. The rats shall be 7 to 8 weeks old and weigh 180 to 220 grams. Prior to testing, the animals shall be selected at random for each group. Animals showing signs of ill health shall not be used.

(iii) *Animal care.* (A) The animals should be housed in environmentally controlled rooms with 10 to 15 air changes per hour. The rooms should be maintained at a temperature of 25 ± 2°C and humidity of 50 ±10 percent with a 12-hour light/dark cycle per day. The rats should be isolated for at least 7 days prior to use.

(B) During the acclimatization period, the rats should be housed in cages on hardwood chip bedding. All animals shall be provided with conventional laboratory diets and water ad libitum.

(2) *Administration of DGBE and DGBA*—(i) *Test substances.* These studies require the use of $^{14}$C-labeled DGBE and DGBA. The use of $^{14}$C-DGBE and $^{14}$C-DGBA is required for the determinations in paragraphs (a) (1), (2), and (3) of this section because they will facilitate the work and improve the reliability of quantitative determinations.

(ii) *Dosage and treatment.* (A) Two doses shall be used in the study, a "low" dose and a "high" dose. When administered dermally, the "high" dose level should ideally induce some overt toxicity such as weight loss. The "low" dose level should correspond to a no observed effect level.

(B) For dermal treatment, the doses shall be applied in a volume adequate to deliver the prescribed doses. The backs of the rats should be lightly shaved with an electric clipper shortly before treatment. The dose shall be applied with a micropipette on a specific area (for example, 2 cm$^2$) on the freshly shaven skin. The dosed areas shall be occluded with an aluminium foil patch which is secured in place with adhesive tape.

(iii) *Washing efficiency study.* Before initiation of the dermal absorption studies described in paragraph (b)(2)(iv)(A) of this section, an initial washing efficiency experiment shall be performed to assess the extent of removal of the applied DGBE and DGBA by washing with soap and water. Groups of four rats should be lightly anesthetized with sodium pentobarbital. These animals shall then be treated with dermal doses of test substance at the low dose level. Soon after application (5 to 10 minutes) the treated animals shall be washed with soap and water then housed in individual metabolism cages for excreta collection. Urine and feces shall be collected at 8, 24, and 48 hours following dosing. Collection of excreta shall continue every 24 hours if a significant amounts of DGBE, DGBA, or metabolites continue to be eliminated.

(iv) *Determination of absorption, biotransformation, and excretion.* (A) Eight animals shall be dosed once dermally with the low dose of $^{14}$C-DGBE.

(B) Eight animals shall be dosed once dermally with the high dose of $^{14}$C-DGBE.

(C) Eight animals shall be dosed once dermally with the low dose of $^{14}$C-DGBA.

(D) Eight animals shall be dosed once dermally with the high dose of $^{14}$C-DGBA.

(E) The high and low doses of $^{14}$C-DGBE and $^{14}$C-DGBA shall be kept on the skin for the duration of the study (96 hours). After application, the animals shall be placed in metabolism cages for excreta collection. Urine and feces shall be collected at 8, 24, 48, 72 and 96 hours after dosing, and if necessary, daily thereafter until at least 90 percent of the dose has been excreted or until 7 days after dosing (whichever occurs first).

(3) *Observation of animals*—(i) *Urinary and fecal excretion.* The quantities of total $^{14}$C excreted in urine and feces by rats dosed as specified in paragraph (b)(2)(iv) of this section shall be determined at 8, 24, 48, 72 and 96 hours after dosing, and if necessary, daily thereafter until at least 90 percent of the dose has been excreted or until 7 days after dosing (whichever occurs first). Four animals from each group shall be used for this purpose.

(ii) *Biotransformation after dermal dosing.* Appropriate qualitative and quantitative methods shall be used to assay urine specimens collected from rats dosed with DGBE as specified in paragraph (b)(2)(iv) of this section. Any metabolite which comprises greater than 10 percent of the dose shall be identified.

(c) *Data and reporting*—(1) *Treatment of results.* Data shall be summarized in tabular form.

(2) *Evaluation of results.* All observed results, quantitative or inciden-

tal, shall be evaluated by an appropriate statistical method.

(3) *Test report.* In addition to the reporting requirements as specified in the TSCA Good Laboratory Practice Standards, in Part 792, Subpart J of this chapter, the following specific information shall be reported:

(i) Species, strain, and supplier of laboratory animals.

(ii) Information on the degree (i.e., specific activity for a radiolabel) and sites of labeling of the test substances.

(iii) A full description of the sensitivity and precision of all procedures used to produce the data.

(iv) Relative percent absorption by the dermal route for rats administered low and high doses of $^{14}$C-DGBE and $^{14}$C-DGBA.

(v) Quantity of isotope, together with percent recovery of the administered dose, in feces and urine.

(vi) Biotransformation pathways and quantities of DGBE and metabolites in urine collected after administering single high and low dermal doses to rats.

[53 FR 5946, Feb. 26, 1988]

### § 795.230 Oral and inhalation pharmacokinetic test.

(a) *Purpose.* The purpose of these studies is to determine:

(1) Bioavailability of test substance after oral and inhalation exposure.

(2) Whether or not the biotransformation of the test substance is qualitatively and quantitatively the same after oral and inhalation exposure.

(3) Whether or not the biotransformation of the test substance is changed qualitatively or quantitatively by repeated dosing.

(b) *Definitions.* Bioavailability refers to the rate and extent to which an administered chemical substance compound is absorbed, i.e., reaches the systemic circulation.

(c) *Test procedures*—(1) *Animal selection*—(i) *Species.* The preferred species is the rat for which extensive data on the toxicity and carcinogenicity of numerous chemical substances are available.

(ii) *Animals.* Adult male and female Fischer 344 rats shall be used. The rats shall be 7 to 9 weeks old. Prior to testing, the animals are selected at random for each group. Animals showing signs of ill health shall not be used.

(iii) *Animal care.* Animals shall be housed in environmentally controlled rooms with 10 to 15 air changes per hour. The rooms shall be maintained at a temperature of 24 ±2 °C and humidity 50 ± 10 percent with a 12-hour light/dark cycle per day. The rats shall be isolated for at least 7 days prior to use, and their health status shall then be evaluated. The animals shall be acclimated to the experimental environment for a minimum of 48 hours prior to treatment. Certified feed and water shall be provided *ad libitum.*

(iv) *Numbers.*—(A) At least 8 animals (4 males and 4 females) shall be used at each dose level.

(B) Females shall be nulliparous and nonpregnant.

(2) *Administration of the test substance*—(i) *Test substance.* The test substance shall be at least 99 percent pure. The studies require the use of both nonradioactive and $^{14}$C-labeled test substance. Both preparations are needed to investigate the provisions of paragraph (a)(2) of this section. The use of $^{14}$C-test substance is recommended for the provisions in paragraphs (a) (1), (2), and (3) of this section in order to facilitate the work, improve the reliability of quantitative determinations, and increase the probability of observing previously unidentified metabolites.

(ii) *Dosage and treatment*—(A) *Oral study.* At least two doses shall be used in the study, a "low" and "high" dose. When administered orally, the "high" dose should induce some overt toxicity such as weight loss. The "low" dose shall not induce observable effects attributable to the test substance. Oral dosing shall be performed by gavage using an appropriate vehicle.

(B) *Inhalation study.* Three concentrations shall be used in the study. Upon exposure, the two higher concentrations should ideally induce some overt symptoms of toxicity, although the intermediate concentration may be excluded from this condition. The lowest concentration shall not induce observable effects attributable to the test substance.

(iii) *Determination of bioavailability*—(A) *Oral studies.* (*1*) Group A (a minimum of 8 animals, 4 males and 4 females) shall be dosed once *per os* with the low dose of $^{14}$C-labeled test substance.

(*2*) Group B (a minimum of 8 animals, 4 males and 4 females) shall be dosed once *per os* with the high dose of $^{14}$C-labeled test substance.

(B) *Inhalation studies.* (*1*) Group C (a minimum of 8 animals, 4 males and 4 females) shall be exposed (6 hours) to a mixture of non-radioactive test substance in air at the prescribed low test substance concentration.

(*2*) Group D (a minimum of 8 animals, 4 males and 4 females) shall be exposed (6 hours) to a mixture of non-radioactive test substance in air at the prescribed intermediate test substance concentration.

(*3*) Group E (a minimum of 8 animals, 4 males and 4 females) shall be exposed (6 hours) to a mixture of non-radioactive test substance in air at the prescribed high concentration.

(*4*) Group F (a minimum of 8 animals, 4 males and 4 females) shall be exposed (6 hours) to a mixture of $^{14}$C-labeled test substance in air at the prescribed low test substance concentration.

(*5*) Group G (a minimum of 8 animals, 4 males and 4 females) shall be exposed (6 hours) to a mixture of $^{14}$C-labeled test substance in air at the prescribed intermediate test substance concentration.

(*6*) Group H (a minimum of 8 animals, 4 males and 4 females) shall be exposed (6 hours) to a mixture of $^{14}$C-labeled test substance in air at the prescribed high test substance concentration.

(C) *Collection of excreta.* After oral administration (Groups A and B) and inhalation exposure (Groups F through H) the rats shall be placed in individual metabolic cages and excreta (urine, feces and expired air) shall be collected from 0 to 24 hours and then from 24 to 48 hours post-treatment, or until 90 percent of the dose has been excreted, whichever occurs first.

(D) *Kinetic studies.* Groups C through E shall be used to determine the concentration of the test substance in blood at 0, 5, 10, 15, and 30 minutes, and at 1, 2, 4, 8, 16, 24, and 48 hours after initiation of inhalation exposure.

(E) *Repeated dosing study.* Rats (a minimum of 8 animals, 4 males and 4 females) shall receive a series of single daily oral doses of non-radioactive test substance over a period of at least 7 days, followed at 24 hours after the last dose by a single oral dose of $^{14}$C-labeled test substance. Each dose shall be at the low-dose level. Urine shall be collected from 0 to 24 hours and then 24 to 48 hours after administering the $^{14}$C-labeled test substance.

(3) *Observation of animals*—(i) *Bioavailability*—(A) *Blood levels.* The levels of total $^{14}$C-label shall be determined in whole blood, blood plasma, or blood serum of each rat at 0, 4, 8, 16, 24, and 48 hours after dosing rats in Groups A-B and F-H.

(B) *Expired air, urinary and fecal excretion.* The quantities of total $^{14}$C-label eliminated in expired air, urine, and feces by each rat in Groups A and B and F through H shall be determined in collections made from 0 to 24 hours and then 24 to 48 hours after dosing and, if necessary, daily thereafter until at least 90 percent of the dose has been excreted or until 7 days after dosing, whichever occurs first.

(C) *Tissue distribution.* The concentration and quantity of $^{14}$C-label in tissue and organs shall be determined at the time of sacrifice for each rat in Groups A and B, F through H, and the repeated-dosing group.

(ii) *Biotransformation after oral and inhalation exposure.* Appropriate qualitative and quantitative methods shall be used to assay urine specimens collected from each rat in Groups A and B and F through H. Suitable enzymatic steps should be used to distinguish, characterize, and quantify conjugated and unconjugated metabolites of the test substance.

(iii) *Change(s) in biotransformation.* Appropriate qualitative and quantitative assay methodologies shall be used to compare the composition of $^{14}$C-labeled components of urine collected from 0 to 24 and then from 24 to 48 hours after dosing rat Group A with those components in the urine collected over the same intervals after administering the radioactive dose in the repeated dosing study.

(d) *Data and reporting*—(1) *Treatment of results.* Data should be summarized in tabular form.

(2) *Evaluation of results.* All observed results shall be evaluated by an appropriate statistical method.

(3) *Test report.* In addition to the reporting requirements as specified in the EPA Good Laboratory Practice Standards (Subpart J, Part 792 of this chapter) the following specific information should be reported:

(i) Labeling site of the test substance.

(ii) A full description of the sensitivity and precision of all procedures used to produce the data.

(iii) Quantity of isotope, together with percent recovery of the administered dose in feces, urine, expired air, and blood for both routes of administration.

(iv) Quantity and distribution of $^{14}$C-test substance in bone, brain, fat, gonads, heart, kidney, liver, lung, muscle, spleen, tissue which displayed pathology, and residual carcass.

(v) Biotransformation pathways and quantities of test substance and its metabolites in urine, feces, and expired air collected after oral administration (single low and high doses) and inhalation exposure (low, intermediate, and high concentrations).

(vi) Biotransformation pathways and quantities of the test substance and its metabolites in urine collected after repeated administration of test substance to rats.

(vii) Pharmacokinetic model(s), if any, developed from the experimental data.

(4) *Counting efficiency.* Data should be made available to the Agency upon request.

[52 FR 37143, Oct. 5, 1987]

## § 795.235 Toxicokinetic Test.

(a) *Purpose.* These studies are designed to: (1) Determine the bioavailability of the test substance after dermal or oral treatment.

(2) Ascertain whether the metabolites of the test substance are similar after dermal (assuming significant penetration) and oral administration.

(3) Examine the effects of a multiple dosing regimen on the metabolism of the test substance after *per os* administration.

(b) *Definition of scope of study.* Absorption toxicokinetics refers to the bioavailability, i.e., the rate and extent of absorption of the test substance, and metabolism and excretion rates of the test substance after absorption.

(c) *Test procedures*—(1) *Animal selection*—(i) *Species.* The rat is the animal species of choice since it has been used extensively for absorption, metabolism, and toxicological studies.

(ii) *Rat strain.* Adult male and female Fischer 344 rats shall be used. At 7 to 9 weeks of age, the males should weigh 125 to 175 g and the females 110 to 150 g. The rats shall be purchased from a reputable dealer and identified with ear tags upon arrival. The animals shall be randomly selected for the testing groups, and no unhealthy animal is to be used for experimentation.

(iii) *Animal care.* (A) Animal care and housing should be in accordance with Department of Health, Education and Welfare Publication No. (NIH)-78-23, 1978. "Guidelines for the Care and Use of Laboratory Animals," or its equivalent.

(B) The animals shall be housed in environmentally controlled rooms with 10 to 15 air changes per hour. The rooms shall be maintained at a temperature of 25±2 °C and humidity of 50±10 percent with a 12-hour light/dark cycle per day. The rats shall be kept in a quarantine facility for at least 7 days prior to use.

(C) During the acclimatization period, the rats shall be housed in polycarbonate cages on hardwood chip bedding. All animals shall be provided with certified feed and tap water *ad libitum.*

(iv) *Number of animals.* There shall be at least four animals of each sex in each experimental group.

(2) *Administration of test substance*—(i) *Test substance.* Test substance of at least 99 percent purity, commercially available, should be used as the test substance. Since both nonradioactive and radioactive (uniformly $^{14}$C-labelled) test substances are to be used, they should be chromatographed

separately and analyzed together, to ascertain purity and identity. The use of $^{14}$C-labelled test substance, diluted with unlabeled test substance, is required for all of the studies under this section, unless otherwise specified, as it will greatly increase the reliability and sensitivity of the quantitative assays and facilitate the identification of metabolites.

(ii) *Dosage and treatment.* (A) Two doses shall be used in studies under this section, a "low" dose and a "high" dose. When administered orally, the "high" dose level should ideally induce some overt toxicity, such as weight loss. The "low" dose level should not induce observable effects attributable to the test substance. If feasible, the same "high" and "low" doses should be administered orally and dermally.

(B) Oral dosing shall be accomplished by gavage after dissolving the test substance in a suitable vehicle. For dermal treatment, the doses shall be administered in a suitable solvent and applied at a volume adequate to deliver the prescribed doses. The backs of the rats should be shaved with an electric clipper one day before treatment. The dose should be applied with a disposable micropipette on a specific area (2 cm$^2$ for rats) on the shaven skin. The dosed areas shall be occluded with an aluminum foil patch which is secured in place with adhesive tape.

(iii) *Determination of test substance kinetics.* Each experimental group shall contain at least four rats of each sex for a total of eight rats.

(A) *Oral studies.* (*1*) Group A shall be dosed once *per os* with the low dose of the test substance.

(*2*) Group B shall be dosed once *per os* with the high dose of the test substance.

(*3*) For the oral studies, the rats shall be placed in individual metabolic cages to facilitate collection of urine and feces at 8, 24, 48, 72, and 96 hours following administration. The cages shall be cleaned at each time period to collect any metabolites that might adhere to the metabolic cages.

(B) *Dermal Studies.* (*1*) Group C shall be dosed once dermally with the low dose of test substance.

(*2*) Group D shall be dosed once dermally with the high dose of test substance.

(*3*)(*i*) For the dermal studies, the test substance shall be applied for 24 hours. Immediately after application, each animal shall be placed in a separate metabolic cage for excreta collection. At the time of removal of the aluminum foil, the occluded area shall be washed with an appropriate solvent (see below), to remove any test substance that may be on the skin surface and the wash solvent assayed for the amount of test substance recovered. At the termination of the experiments, each animal shall be sacrificed and the exposed skin area removed. The skin (or an appropriate section) shall be solubilized and assayed for the test substance and its metabolites.

(*ii*) Before initiation of the dermal studies, an initial washing efficiency experiment shall be conducted to assess the removal of the applied test substance by washing the exposed skin area with soap and water or organic solvents. Four rats, two of each sex, shall be lightly anesthetized and then test substance applied to a specified area. After application (5 to 10 minutes), the areas should be washed with soap and water (two rats) or ethanol and water (two rats). The amount recovered shall be determined to assess efficacy of test substance removal by washing of the skin.

(C) *Repeated dosing study group E.* Four rats (two of each sex) shall receive a series of single daily oral doses of nonradioactive test substance over a period of at least 14 days, followed at 24 hours after the last dose by a single oral dose of $^{14}$C-labelled test substance. Each dose shall be at the low dose level.

(3) *Observation of animals*—(i) *Bioavailability*—(A) *Blood levels.* The levels of $^{14}$C shall be determined in whole blood, blood plasma, or blood serum at appropriate intervals from 1 to 96 hours after dosing rats in Groups A through E. Four rats (two of each sex) of each group shall be used for this purpose.

(B) *Urinary and fecal excretion.* The quantities of $^{14}$C excreted in the urine and feces by rats in Groups A through E shall be determined at 8 hours, 24 hours, 48 hours, 72 hours, and 96 hours after dosing, and if necessary, daily thereafter until at least 90 percent of the applied dose has been excreted or until 7 days after dosing (whichever occurs first). Four animals (two of each sex) shall be used for these analyses.

(ii) *Biotransformation after oral and dermal dosing.* Appropriate qualitative and quantitative methods shall be used to assay the test substance and metabolites in the urine and fecal specimens collected from rat Groups A through D.

(iii) *Changes in Biotransformation.* Appropriate qualitative and quantitative assay methodology shall be used to compare the composition of $^{14}$C-labelled compounds in excreta collected at 14 and 48 hours after dosing rat Group A with those in the excreta collected at 24 and 48 hours after the $^{14}$C-labelled test substance dose in the repeated dose study (Group E).

(d) *Data and reporting*—(1) *Treatment of results.* Data should be summarized in tabular form.

(2) *Evaluation of results.* All observed results, quantitative or incidental, should be evaluated by an appropriate statistical method.

(3) *Test report.* In addition to the reporting requirements specified in the EPA Good Laboratory Practice Standards (40 CFR Part 792, Subpart J) the following specific information shall be reported:

(i) Specie(s) and strain(s) of laboratory animals.

(ii) Information on the degree (i.e., specific activity for a radiolabel) and site(s) of labeling of the test substance;

(iii) A full description of the sensitivity and precision of all procedures used to produce the data.

(iv) Percent absorption by oral and dermal routes of rats administered $^{14}$C-test substance.

(v) Quantity of isotope, together with percent recovery of administered dose in feces, urine, blood, and skin and skin washings (dermal study only for last two portions).

(vi) Quantity and distribution of $^{14}$C-labelled test substance in various tissues, including bone, brain, fat, gonads, heart, kidney, liver, lung, muscle, spleen, and residual carcass.

(vii) Counting efficacy data shall be made available to the Agency upon request.

[52 FR 19868, May 28, 1987]

### § 795.250 Developmental neurotoxicity screen.

(a) *Purpose.* In the assessment and evaluation of the toxic characteristics of a chemical, it is important to determine when acceptable exposures in the adult may not be acceptable to a developing organism. This test is designed to provide information on the potential functional and morphologic hazards to the nervous system which may arise in the offspring from exposure of the mother during pregnancy and lactation.

(b) *Principle of the test method.* The test substance is administered to several groups of pregnant animals during gestation and lactation, one dose level being used per group. Offspring are randomly selected from within litters for neurotoxicity evaluation. The evaluation includes observation to detect gross neurological and behavioral abnormalities, determination of motor activity, neuropathological evaluation, and brain weights. Measurements are carried out periodically during both postnatal development and adulthood.

(c) *Test procedures*—(1) *Animal selection*—(i) *Species and strain.* Testing should be performed in the Sprague Dawley rat.

(ii) *Age.* Young adult animals (nulliparous females) shall be used.

(iii) *Sex.* Pregnant females shall be used at each dose level.

(iv) *Number of animals.* The objective is for a sufficient number of pregnant rats to be exposed to ensure that an adequate number of offspring are produced for neurotoxicity evaluation. At least 20 litters are recommended at each dose level. This number assumes a coefficient of variation of 20 to 25 percent for most behavioral tests. If, based upon experience with historical control data or data for positive controls in a given laboratory, the coefficient of variation for a given task is higher than 20 to 25 percent, then calculation of appropriate sample sizes to detect a 20 percent change from control values with 80 percent power would need to be done. For most designs, calculations can be made according to Dixon and Massey (1957) under paragraph (e)(5) of this section, Neter and Wasserman (1974) under paragraph (e)(10) of this section, Sokal and Rohlf (1969) under paragraph (e)(11) of this section, or Jensen (1972) under paragraph (e)(8) of this section.

(A) On day 4 after birth, the size of each litter should be adjusted by eliminating extra pups by random selection to yield, as nearly as possible, 4 males and 4 females per litter. Whenever the number of male or female pups prevents having 4 of each sex per litter, partial adjustment (for example, 5 males and 3 females) is permitted. Adjustments are not appropriate for litters of less than 8 pups. Elimination of runts only is not appropriate. Individual pups should be identified uniquely after standardization of litters. A method that may be used can be found in Adams et al. (1985) under paragraph (e)(1) of this section.

(B) After standardization of litters, males and females shall be randomly assigned to one of each of three behavioral tasks. Alternatively, more than one of the behavioral tasks may be conducted in the same animal. In the latter case, a minimum of 1 to 2 days should separate the tests when conducted at about the same age.

(C) One male and one female shall be randomly selected from each litter for sacrifice at weaning as specified in paragraph (c)(8) of this section.

(2) *Control group.* A concurrent control group shall be used. This group shall be a sham treated group, or, if a vehicle is used in administering the test substance, a vehicle control group. Animals in the control groups shall be handled in an identical manner to test group animals. The vehicle shall neither be developmentally toxic nor have effects on reproduction.

(3) *Dose levels and dose selection.* (i) At least 3 dose levels plus a control (vehicle control, if a vehicle is used) shall be used.

(ii) If the substance has been shown to be developmentally toxic either in a standard developmental toxicity study or a pilot study, the highest dose level shall be the maximum dose which will not induce *in utero* or neonatal deaths or malformations sufficient to preclude a meaningful evaluation of neurotoxicity.

(iii) In the absence of standard developmental toxicity, unless limited by the physicochemical nature or biological properties of the substance, the highest dose level shall induce some overt maternal toxicity but shall not result in a reduction in weight gain exceeding 20 percent during gestation and lactation.

(iv) The lowest dose should not produce any grossly observable evidence of either maternal or developmental neurotoxicity.

(v) The intermediate dose(s) shall be equally spaced between the highest and lowest dose.

(4) *Dosing period.* Day 0 in the test is the day on which a vaginal plug and/or sperm are observed. The dose period shall cover the period from day 6 of gestation through weaning (21 days postnatally).

(5) *Administration of test substance.* The test substance or vehicle should be administered orally by intubation. The test substance shall be administered at the same time each day. The animals shall be weighed periodically and the dosage based on the most recent weight determination.

(6) *Observation of dams.* (i) A gross examination of the dams shall be made at least once each day, before daily treatment. The animals shall be observed by trained technicians who are blind with respect to the animal's treatment, using standardized procedures to maximize inter-observer reliability. Where possible, it is advisable that the same observer be used to evaluate the animals in a given study. If this is not possible, some demonstration of inter-observer reliability is required.

(ii) During the treatment and observation periods, cage-side observations shall include:

(A) Any responses with respect to body position, activity level, coordination of movement, and gait.

(B) Any unusual or bizarre behavior including, but not limited to headflicking, head searching, compulsive biting or licking, self-mutilation, circling, and walking backwards.

(C) The presence of:

*(1)* Convulsions.

*(2)* Tremors.

*(3)* Increased levels of lacrimation and/or red-colored tears.

*(4)* Increased levels of salivation.

*(5)* Piloerection.

*(6)* Pupillary dilation or constriction.

*(7)* Unusual respiration (shallow, labored, dyspneic, gasping, and retching) and/or mouth breathing.

*(8)* Diarrhea.

*(9)* Excessive or diminished urination.

*(10)* Vocalization.

(iii) Signs of toxicity shall be recorded as they are observed, including the time of onset, the degree and duration.

(iv) Animals shall be weighed at least weekly.

(v) The day of delivery of litters shall be recorded.

(7) *Study conduct*—(i) *Observation of offspring.* (A) All offspring shall be examined cage-side daily for gross signs of mortality and morbidity.

(B) All offspring shall be examined outside the cage for gross signs of toxicity whenever they are weighed or removed from their cages for behavioral testing. The offspring shall be observed by trained technicians, who are blind with respect to the animal's treatment using standardized procedures to maximize inter-observer reliability. Where possible, it is advisable that the same observer be used to evaluate the animals in a given study. If this is not possible, some demonstration of inter-observer reliability is required. At a minimum, the end points outlined in paragraph (c)(6)(ii) of this section shall be monitored as appropriate for the developmental stage being observed.

(C) Any gross signs of toxicity in the offspring shall be recorded as they are observed, including the time of onset, the degree, and duration.

(ii) *Developmental landmarks.* Live pups should be counted and litters weighed by weighing each individual pup at birth, or soon thereafter, and on days 4, 7, 13, 17, and 21, and biweekly thereafter. The age of the pups at the time of the appearance of the following developmental landmarks shall be determined:

(A) *Vaginal opening.* General procedure for this determination may be found in Adams et al. (1985) under paragraph (e)(1) of this section.

(B) *Testes descent.* General procedure for this determination may be found in Adams et al. (1985) under paragraph (e)(1) of this section.

(iii) *Motor activity.* (A) Motor activity shall be monitored specifically on days 13, 17, 21, 45 (±2 days), and 60 (±2 days). Motor activity shall be monitored by an automated activity recording apparatus. The device used shall be capable of detecting both increases and decreases in activity, i.e., baseline activity as measured by the device shall not be so low as to preclude decreases nor so high as to preclude increases. Each device shall be tested by standard procedures to ensure, to the extent possible, reliability of operation across devices and testing of animals within dose groups shall be balanced across devices.

(B) Each animal shall be tested individually. The test session shall be long enough to demonstrate habituation of motor activity in control animals, i.e., to approach asymptotic levels by the last 20 percent of the session. Animals' activity counts shall be collected in equal time periods of no greater than 10 minutes duration. All sessions shall have the same duration. Treatment groups shall be counter-balanced across test times.

(C) Efforts shall be made to ensure that variations in the test conditions are minimal and are not systematically related to treatment. Among the variables which can affect motor activity are sound level, size, and shape of the test cage, temperature, relative humidity, lighting conditions, odors, use of home cage or novel test cage, and environmental distractions.

(D) Additional information on the conduct of a motor activity study may be obtained in the TSCA motor activity guideline, in § 798.6200 of this chapter.

(iv) *Auditory startle test.* An auditory startle habituation test shall be performed on the offspring on days 22 and 60. Details on the conduct of this testing may be obtained in Adams et al. (1985) under paragraph (e)(1) of this section. In performing the auditory startle task, the mean response amplitude on each block of 10 trials (5 blocks of 10 trials per session on each day of testing) shall be made. While use of pre-pulse inhibition is not a requirement, it may be used at the discretion of the investigator. Details on the conduct of this testing may be obtained from Ison (1984) under paragraph (e)(7) of this section.

(v) *Active avoidance test.* Active avoidance testing shall be conducted beginning at 60 to 61 days of age. Details on the apparatus may be obtained in Brush and Knaff (1959) and on the conduct of testing from Brush (1962), under paragraphs (e)(2) and (e)(4) of this section, respectively; reviews on active avoidance conditioning by Brush (1971) and McAllister and McAllister (1971) can be found under paragraphs (e)(3) and (e)(9) of this section, respectively. In performing the active avoidance task, the following measures should be made:

(A) Mean number of shuttles during the adaptation period preceding each daily session.

(B) Mean number and latency of avoidances per session, presented in blocks of 10 trials (2 blocks of 10 trials per session across 5 sessions).

(C) Mean number and latency of escapes per session, presented in blocks of 10 trials as above.

(D) Mean duration of shocks per session, presented in blocks of 10 trials as above

(E) Mean number of shuttles during the inter-trial intervals.

(8) *Post-mortem evaluation*—(i) *Age of animals.* One male and one female per litter shall be sacrificed at weaning and the remainder following the last behavioral measures. Neuropathology and brain weight determinations shall be made on animals sacrificed at weaning and after the last behavioral measures.

(ii) *Neuropathology.* Details for the conduct of neuropathology evaluation may be obtained in the TSCA neuropathology guideline, in § 798.6400 of this chapter. At least 6 offspring per dose group shall be randomly selected from each sacrificed group (weaning and adulthood) for neuropathologic evaluation. These animals shall be balanced across litters, and equal numbers of males and females shall be used. The remaining sacrificed animals shall be used to determine brain weight. Animals shall be perfused *in situ* by a generally recognized technique. After perfusion, the brain and spinal cord shall be removed and gross abnormalities noted. Cross-sections of the following areas shall be examined: The forebrain, the center of the cerebrum and midbrain, the cerebellum and pons, and the medulla oblongata; the spinal cord at cervical and lumbar swelling; Gasserian ganglia, dorsal root ganglia, dorsal and ventral root fibers, proximal sciatic nerve (midthigh and sciatic notch); sural nerve (at knee), and tibial nerve (at knee). Tissue samples from both the central and peripheral nervous system shall be further immersion-fixed and stored in appropriate fixative for further examination. After dehydration, tissue specimens shall be cleared with xylene and embedded in paraffin or paraplast except for the sural nerve which should be embedded in plastic. A method for plastic embedding is described by Spencer et al. under paragraph (e)(12) of this section. Tissue sections shall be prepared from the tissue blocks. The following general testing sequence is recommended for gathering histopathological data:

(A) *General staining.* A general staining procedure shall be performed on all tissue specimens in the highest treatment group. Hematoxylin and eosin (H&E) shall be used for this purpose. The staining shall be differentiated properly to achieve bluish nuclei with pinkish background.

(B) *Special stains.* Based on the results of the general staining, selected sites and cellular components shall be further evaluated by use of specific techniques. If H&E screening does not provide such information, a battery of stains shall be used to assess the following components in all appropriate required samples: Neuronal body (e.g., Einarson's gallocyanin), axon (e.g., Kluver's Luxol Fast Blue), and neurofibrils (e.g., Bielchosky). In addition, nerve fiber teasing shall be used. A section of normal tissue shall be included in each staining to assure that adequate staining has occurred. Any changes shall be noted and representative photographs shall be taken. If lesions are observed, the special techniques shall be repeated in the next lower treatment group until no further lesions are detectable.

(C) *Alternative technique.* If the anatomical locus of expected neuropathology is well-defined, epoxy-embedded sections stained with toluidine blue may be used for small sized tissue samples. This technique obviates the need for special stains.

(iii) *Brain weight.* At least 10 animals that are not sacrificed for histopathology shall be used to determine brain weight. The animals shall be decapitated and the brains carefully removed, blotted, chilled, and weighed. The following dissection shall be performed on an ice-cooled glass plate: First, the rhombencephalon is separated by a transverse section from the rest of the brain and dissected into the cerebellum and the medulla oblongata/pons. A transverse section is made at the level of the "optic chiasma" which delimits the anterior part of the hypothalamus and passes through the anterior commissure. The cortex is peeled from the posterior section and added to the anterior section. This divides the brain into four sections, the telencephalon, the diencephalon/mid-brain, the medulla oblongata/pons, and the cerebellum. Sections shall be weighed as soon as possible after dissection to avoid drying. Detailed methodology is available in Glowinski and Iversen (1966) under paragraph (e)(6) of this section.

(d) *Data reporting and evaluation.* In addition to the reporting requirements specified in Part 792, Subpart J of this chapter, the final test report shall include the following information.

(1) *Description of system and test methods.* (i) A detailed description of the procedures used to standardize observation and operational definitions for scoring observations.

(ii) Positive control data from the laboratory performing the test that demonstrate the sensitivity of the procedures being used. These data do not have to be from studies using prenatal exposures. However, the laboratory must demonstrate competence in testing neonatal animals perinatally exposed to chemicals and establish test norms for the appropriate age group.

(iii) Procedures for calibrating and assuring the equivalence of devices and balancing treatment groups.

(iv) A short justification explaining any decisions where professional judgement is involved such as fixation technique and choice of stains.

(2) *Results.* The following information shall be arranged by test group dose level.

(i) In tabular form, data for each animal shall be provided showing:

(A) Its identification number and litter from which it came.

(B) Its body weight and score on each developmental landmark at each observation time; total session activity counts and intrasession subtotals on each day measured; auditory startle response magnitude session counts and intrasession subtotals on each day measured; avoidance session counts and intrasession counts on each day measured; time and cause of death (if appropriate); locations, nature or frequency, and severity of the lesions; total brain weight; absolute weight of each of the four sections; and weight of each section as a percentage of total brain weight. A commonly used scale such as 1+, 2+, 3+, and 4+ for degree of severity of lesions ranging from very slight to extensive may be used for morphologic evaluation. Any diagnoses derived from neurologic signs and lesions, including naturally occurring diseases or conditions, shall also be recorded.

(ii) Summary data for each group shall include:

(A) The number of animals at the start of the test.

(B) Body weights of the dams during gestation and lactation.

(C) Litter size and mean weight at birth.

(D) The number of animals showing each observation score at each observation time.

(E) The percentage of animals showing each abnormal sign at each observation time.

(F) The mean and standard deviation for each continuous end point at each observation time. These will include body weight, motor activity counts, acoustic startle responses, performance in active avoidance tests, and brain weights (both absolute and relative).

(G) The number of animals in which any lesion was found.

(H) The number of animals affected by each different type of lesion, the average grade of each type of lesion, and the frequency of each different type and/or location of lesions.

(3) *Evaluation of data.* An evaluation of the test results shall be made. The evaluation shall include the relationship between the doses of the test substance and the presence or absence, incidence, and severity of any neurotoxic effect. The evaluation shall include appropriate statistical analyses. The choice of analyses shall consider tests appropriate to the experimental design and needed adjustments for multiple comparisons.

(e) *References.* For additional background information on this test guideline, the following references should be consulted:

(1) Adams, J., Buelke-Sam, J., Kimmel, C.A., Nelson, C.J., Reiter, L.W., Sobotka, T.J., Tilson, H.A., and Nelson, B.K. "Collaborative behavioral teratology study: Protocol design and testing procedure." *Neurobehavioral Toxicology and Teratology.* 7: 579-586. (1985).

(2) Brush, F.R. "The effects of intertrial interval on avoidance learning in the rat." *Journal of Comparative Physiology and Psychology.* 55: 888-892. (1962).

(3) Brush, F.R. "Retention of aversively motivated behavior." In: "Adverse Conditioning and Learning." Brush, F.R., ed., New York: Academic Press. (1971).

(4) Brush, F.R. and Knaff, P.R. "A device for detecting and controlling automatic programming of avoidance-conditioning in a shuttle-box." *American Journal of Psychology.* 72: 275-278 (1959).

(5) Dixon, W.J. and Massey, E.J. "Introduction to Statistical Analysis." 2nd ed. New York: McGraw-Hill. (1957).

(6) Glowinski, J. and Iversen, L.L. "Regional studies of catecholamines in the rat brain-I." *Journal of Neurochemistry.* 13: 655-669. (1966).

(7) Ison, J.R. "Reflex modification as an objective test for sensory processing following toxicant exposure." *Neurobehavioral Toxicology and Teratology.* 6: 437-445. (1984).

(8) Jensen, D.R. "Some simultaneous multivariate procedures using Hotelling's T2 Statistics." *Biometrics.* 28: 39-53. (1972).

(9) McAllister, W.R. and McAllister, D.E. "Behavioral measurement of conditioned fear." In: "Adverse Conditioning and Learning." Brush, F.R., ed, New York: Academic Press (1971).

(10) Neter, J. and Wasserman, W. "Applied Linear Statistical Models." Homewood: Richard D. Irwin, Inc. (1974).

(11) Sokal, R.P. and Rohlf, E.J. "Biometry." San Francisco: W.H. Freeman and Co. (1969).

(12) Spencer, P.S., Bischoff, M.C., and Schaumburg, H.H., "Neuropathological methods for the detection of neurotoxic disease." In: "Experimental and Clinical Neurotoxicology." Spencer, P.S. and Schaumburg, H.H., eds, Baltimore, MD: Williams & Wilkins, pp. 743-757. (1980).

[53 FR 5957, Feb. 26, 1988]

### § 795.260 Subchronic oral toxicity test.

(a) *Purpose.* In the assessment and evaluation of the toxic characteristics of a test substance, the determination of subchronic oral toxicity may be carried out after initial information on toxicity has been obtained by acute testing. The subchronic oral study has been designed to permit the determination of the no-observed-effect level and toxic effects associated with continuous or repeated exposure to a test substance for a period of 90 days. The test is not capable of determining those effects that have a long latency period for development (e.g., carcinogenicity and life shortening). It provides information on health hazards likely to arise from repeated exposure by the oral route over a limited period of time. It will provide information on target organs, the possibilities of accumulation, and can be of use in selecting dose levels for chronic studies and for establishing safety criteria for human exposure.

(b) *Definitions.* (1) Subchronic oral toxicity is the adverse effects occurring as a result of the repeated daily exposure of experimental animals to a chemical for a part (approximately 10 percent for rats) of a life span.

(2) Dose is the amount of test substance administered. Dose is expressed as weight of test substance (g, mg) per unit weight of test animal (e.g., mg/kg), or as weight of test substance per unit weight of food or drinking water.

(3) No-effect level/No-toxic-effect level/No-adverse-effect level/No-observed-effect level is the maximum dose used in a test which produces no observed adverse effects. A no-observed-effect level is expressed in terms of the weight of a substance given daily per unit weight of test animal (mg/kg). When administered to animals in food or drinking water, the no-observed-effect level is expressed as mg/kg of food of mg/ml of water.

(4) Cumulative toxicity is the adverse effects of repeated doses occurring as a result of prolonged action on, or increased concentration of, the administered substance or its metabolites in susceptible tissue.

(c) *Principle of the test method.* The test substance is administered orally in graduated daily doses to several groups of experimental animals, one dose level per group, for a period of 90 days. During the period of administration the animals are observed daily to detect signs of toxicity. Animals which die during the period of administration are necropsied. At the conclusion of the test all animals are necropsied and histopathological examinations carried out.

(d) *Test procedures*—(1) *Animal selection*—

(i) *Species.* Rats and mice shall be used.

(ii) *Age.* (A) Young adult animals shall be employed. At the commencement of the study the weight variation of animals used shall not exceed ± 20 percent of the mean weight for each sex.

(B) Dosing shall begin as soon as possible after weaning, ideally before the animals are 6 weeks old, and in any case not more than 8 weeks old.

(iii) *Sex.* (A) Equal numbers of animals of each sex should be used at each dose level.

(B) The females should be nulliparous and non-pregnant.

(iv) *Numbers.* (A) At least 20 rats and 20 mice (10 females and 10 males of each species) shall be used at each dose level.

(B) If interim sacrifices are required, the number shall be increased by the number of animals scheduled to be sacrificed before the completion of the study.

(2) *Control groups.* A concurrent control group is required. This group shall be an untreated or sham-treated control group or, if a vehicle is used in administering the test substance, a vehicle control group. If the toxic properties of the vehicle are not known or cannot be made available, both untreated and vehicle control groups are required.

(3) *Satellite group.* A satellite group of 20 rats and 20 mice (10 females and 10 males of each species) shall be treated with the high dose level for 90 days and observed for reversibility, persistence, or delayed occurrence of toxic effects for a post-treatment period of not less than 28 days.

(4) *Dose levels and dose selection.* (i) In subchronic toxicity tests, it is desirable to have a dose response relationship as well as no-observed-toxic-effect level. Therefore, at least three dose levels with a control and, where appropriate, a vehicle control (corresponding to the concentration of vehicle at the highest exposure level) shall be used. Doses should be spaced appropriately to produce test groups with a range of toxic effects. The data shall be sufficient to produce a dose-response curve.

(ii) The highest dose level shall result in toxic effects but not produce an incidence of fatalities which would prevent a meaningful evaluation.

(iii) The lowest dose level shall not produce any evidence of toxicity. Where there is a usable estimation of human exposure the lowest dose level shall exceed this.

(iv) Ideally, the intermediate dose level(s) should produce minimal observable toxic effects. If more than one intermediate dose is used, the dose levels should be spaced to produce a gradation of toxic effects.

(v) The incidence of fatalities in low and intermediate dose groups and in the controls should be low to permit a meaningful evaluation of the results.

(5) *Exposure conditions.* Ideally the animals should be dosed with the test substance on a 7-day per week basis over a period of 90 days. However, based primarily on practical considerations, dosing by gavage or capsule studies on a 5-day per week basis shall be acceptable.

(6) *Observation period.* (i) Duration of observation shall be for at least 90 days.

(ii) Animals in the satellite group scheduled for followup observations shall be kept for not less than 28 days without treatment to detect recovery from, or persistence of, toxic effects.

(7) *Administration of the test substance.* (i) The test substance shall be administered in the diet or in capsules. Alternatively, it may be administered by gavage or in the drinking water.

(ii) All animals shall be dosed by the same method during the entire experimental period.

(iii) Where necessary, the test substance is dissolved or suspended in a suitable vehicle. If a vehicle or diluent is needed, ideally it should not elicit important toxic effects itself nor substantially alter the chemical or toxicological properties of the test substance. It is recommended that wherever possible the usage of an aqueous solution be considered first, followed by consideration of a solution of oil, and then by possible solution in other vehicles.

(iv) For substances of low toxicity, it is important to ensure that when administered in the diet the quantities of the test substance involved do not interfere with normal nutrition. When the test substance is administered in the diet, either a constant dietary concentration (ppm) or a constant dose level in terms of the animals' body weight shall be used; the alternative used shall be specified.

(v) For a substance administered by gavage or capsule, the dose shall be given at similar times each day, and adjusted at intervals (weekly or biweekly) to maintain a constant dose level in terms of animal body weight.

(8) *Observation of animals.* (i) Each animal shall be handled and its physical condition appraised at least once each day.

(ii) Additional observation shall be made daily with appropriate actions taken to minimize loss of animals to the study (e.g., necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals).

(iii) Signs of toxicity shall be recorded as they are observed including the time of onset, degree, and duration.

(iv) Cage-side observations shall include, but not be limited to, changes in skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity, and behavior pattern.

(v) Measurements shall be made weekly of food consumption or water consumption when the test substance is administered in the food or drinking water, respectively.

(vi) Animals shall be weighed weekly.

(vii) At the end of the 90-day period all survivors in the nonsatellite treatment group shall be sacrificed. Moribund animals shall be removed and sacrificed when noticed.

(9) *Clinical examinations.* (i) The following examinations shall be made on at least five animals of each sex in each group of rats.

(A) Certain hematology determinations shall be carried out just prior to terminal sacrifice at the end of the test period. The following hematology determinations shall be carried out: hematocrit, hemoglobin concentration, erythrocyte count, total and differential leucocyte count, and a measure of clotting potential such as clotting time, prothrombin time, thromboplastin time, or platelet count.

(B) Certain clinical biochemistry determinations shall be carried out just prior to terminal sacrifice at the end of the test period. The following clinical biochemical test areas shall be carried out: Electrolyte balance, carbohydrate metabolism, and liver and kidney function. The selection of additional tests shall be influenced by observations on the mode of action of the substance. Suggested additional determinations include: Calcium, phosphorus, chloride, sodium, potassium, fasting glucose (with period of fasting appropriate to the species/breed), serum glutamic-pyruvic transaminase (now known as serum alanine aminotransferase), serum glutamic oxaloacetic transaminase (now known as serum aspartate aminotransferase), ornithine decarboxylase, gamma glutamyl transpeptidase, urea nitrogen, albumen, blood creatinine, total bilirubin, and total serum protein measurements. Other determinations which may be necessary for an adequate toxicological evaluation include analyses of lipids, hormones, acid/base balance, methemoglobin, and cholinesterase activity. Additional clinical biochemistry may be employed where necessary to extend the investigation of observed effects.

(ii) The following examinations shall be made on at least five animals of each sex in each group.

(A) Ophthalmological examination, using an ophthalmoscope or equivalent suitable equipment, shall be made prior to the administration of the test substance and at the termination of the study. If changes in the eyes are detected, all animals shall be examined.

(B) Urinalysis is required only when there is an indication based on expected or observed toxicity.

(10) *Gross necropsy*. (i) All animals shall be subjected to a full gross necropsy which includes examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents.

(ii) At least the liver, kidneys, adrenals, gonads, and brain shall be weighed wet, as soon as possible after dissection to avoid drying.

(iii) The following organs and tissues, or representative samples thereof, shall be preserved in a suitable medium for possible future histopathological examination: All gross lesions; brain—including sections of medulla/pons, cerebellar cortex and cerebral cortex; pituitary; thyroid/parathyroid; thymus; lungs; trachea; heart; sternum with bone marrow; salivary glands; liver; spleen; kidneys/adrenals; pancreas; gonads; uterus; accessory genital organs (epididymis, prostrate, and, if present, seminal vesicles); aorta; (skin), (non-rodent gall bladder); esophagus; stomach; duodenum; jejunum; ileum; cecum; colon; rectum; urinary bladder; representative lymph node; (mammary gland), (thigh musculature), peripheral nerve; (eyes), (femur including articular surface), (spinal cord at three levels—cervical, midthoracic and lumbar); and, (rodent-exorbital lachrymal glands).

(11) *Histopathology*. (i) Full histopathology shall be performed on the organs and tissues, listed under paragraphs (d)(10) (ii) and (iii) of this section of all animals in the control and high-dose groups, and all animals that died or were killed during the study.

(ii) Histopathology shall be performed on all gross lesions in all animals.

(iii) Histopathology shall be performed on target organs in all animals.

(iv) Histopathology shall be performed on the tissues mentioned in brackets under paragraph (d)(10)(iii) of this section if indicated by signs of toxicity or target organ involvement.

(v) Histopathology shall be performed on lungs, liver, and kidneys of all animals. Special attention to examination of the lungs should be made for evidence of infection since this provides a convenient assessment of the state of health of the animals.

(vi) For the satellite group, histopathology shall be performed on tissues and organs identified as showing effects in the treated groups.

(e) *Data and reporting*—(1) *Treatment of results*. (i) Data shall be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions, the type of lesions, and the percentage of animals displaying each type of lesion.

(ii) All observed results, quantitative and incidental, shall be evaluated by an appropriate statistical method. Any generally acceptable statistical methods may be used; the statistical methods should be selected during the design of the study.

(2) *Evaluation of the study results*. (i) The findings of a subchronic oral toxicity study should be evaluated in

conjunction with the findings of preceding studies and considered in terms of the toxic effects and the necropsy and histopathological findings. The evaluation shall include the relationship between the dose of the test substance and the presence or absence, the incidence and severity, of abnormalities, including behavioral and clinical abnormalities, gross lesions, identified target organs, body weight changes, effects on mortality and any other general or specific toxic effects. The test shall provide a satisfactory estimation of a no-effect level.

(ii) In any study which demonstrates an absence of toxic effects, further investigation to establish absorption and bioavailability of the test substance shall be considered.

(3) *Test report.* In addition to the reporting requirements as specified in the TSCA Good Laboratory Practice Standards, Subpart J of Part 792 of this chapter, the following specific information shall be reported:

(i) *Group animal data.* Tabulation of toxic response data by species, strain, sex, and exposure level for:

(A) Number of animals dying.

(B) Number of animals showing signs of toxicity.

(C) Number of animals exposed.

(ii) *Individual animal data.* (A) Time of death during the study or whether animals survived to termination.

(B) Time of observation of each abnormal sign and its subsequent course.

(C) Body weight data.

(D) Food consumption data when collected.

(E) Hematological tests employed and all results.

(F) Clinical biochemistry tests employed and all results.

(G) Necropsy findings.

(H) Detailed description of all histopathological findings.

(I) Statistical treatment of results where appropriate.

[51 FR 40328, Nov. 6, 1986]

### § 795.285 Morphologic transformation of cells in culture.

(a) *Purpose. In vitro* assays for cellular transformation are semi-quantitative assays for the ability of chemical agents to morphologically alter (transform) cells in culture. Such transformation is associated with certain phenotypic changes such as loss of contact inhibition and the ability to form colonies in soft agar medium. The process by which these changes occur is assumed to be closely related to the process of *in vivo* carcinogenesis. Morphologically transformed cells appear as foci of dense, piled-up, altered cells on an underlying monolayer of normal cells. Three types of foci have been recognized. Type III foci appear to be most closely correlated with *in vivo* tumor formation. The ultimate criterion for morphologic transformation is the ability of the transformed cells to induce tumors when inoculated into appropriate hosts. Not all cells which appear to be morphologically transformed are capable of tumor formation. In general, there is reasonably good correlation between *in vitro* transformation and *in vivo* oncogenesis, although the correlation varies depending on the system being studied. These systems are believed to be reasonably good predictors of *in vivo* activity, and positive results are viewed as potential indications of *in vivo* carcinogenesis.

(b) *Definitions.* (1) Morphologic transformation is the acquisition of certain phenotypic characteristics, most notably loss of contact inhibition and loss of anchorage dependence, which are often but not always associated with the ability to induce tumors in appropriate hosts.

(2) Type III foci of transformed cells are multilayered aggregations of densely staining cells with random orientation and criss-cross arrays at the periphery of the aggregate. They appear as dark stained areas on a light staining background monolayer which is one-cell thick.

(c) *Reference substances.* Not applicable.

(d) *Test method*—(1) *Principle.* (i) Three systems for detecting chemically induced morphologic transformation have been described. They are:

(A) Systems which employ cell lines (cells with an indefinite lifespan).

(B) Systems which employ cell strains (cells with a finite or limited lifespan).

(C) Systems which detect the interaction between chemicals and oncogenic viruses.

(ii) This study shall employ an established cell line for detection of morphologic transformaton.

(2) *Description.* Cells in culture are exposed to the test substance, both with and without metabolic activation, for a defined period of time. Cytotoxicity is determined by measuring the colony-forming ability and growth rate of the cultures after the treatment period. At the end of the treatment period, cultures are maintained in growth medium for a sufficient period of time to allow near-optimal expression of transformed foci.

(3) *Cells.* (i) Balb/c-3T3 mouse cells originally obtained from clone A-31 or its derivatives shall be used in the assay. Cells shall be checked for mycoplasm contamination prior to use in the assay and may be checked for karyotype.

(ii) Appropriate culture media and incubation conditions (culture vessels, $CO_2$ concentrations, temperature, and humidity) shall be used.

(4) *Metabolic activation.* Cells shall be exposed to test substance both in the presence and absence of a metabolic activation system. The metabolic activation system shall be derived from primary cultures of rat hepatocytes.

(5) *Control groups.* Positive and negative (untreated and vehicle) controls shall be included in each experiment. 3-Methylcholanthrene is an example of a positive control for experiments without metabolic activation. Dimethylnitrosamine is an example of a positive control in experiments with metabolic activation.

(6) *Test chemicals*—(i) *Vehicle.* Test agents shall be dissolved in serum-complete culture medium prior to treatment of the cells.

(ii) *Exposure concentrations.* Several concentrations (usually at least four) of the test substance shall be used. These shall be selected on the basis of a preliminary cytotoxicity assay performed both with and without metabolic activation. The highest concentration shall produce a low level of survival (approximately 10 to 20 percent), and the survival in the lowest concentration shall approximate that of the negative control.

(e) *Test performance.* (1) Cells shall be exposed to the test substance both with and without metabolic activation. Exposure shall be for 72 hours for experiments without metabolic activation and for 48 hours for experiments with metabolic activation unless different exposure times are justified by the investigator.

(2) At the end of the exposure period, cells shall be washed and cultured to determine viability and to allow for expression of transformation.

(3) At the end of the incubation period (generally 4 to 6 weeks), cells shall be fixed and stained, and the number of transformed (Type III) foci shall be enumerated.

(4) All results shall be confirmed in an independent experiment if a single, statistically significant positive effect is produced at one dose point without a dose response. A positive response should be confirmed by testing over a narrow range of concentrations.

(5) Tumorigenic potential of isolated morphologically transformed foci may be determined by inoculation into suitable hosts.

(f) *Data and report*—(1) *Treatment of results.* (i) Data shall be presented in tabular form. Individual colony counts for the treated and control groups shall be presented for both transformation and survival.

(ii) Survival and cloning efficiencies shall be given as a percentage of the controls. Transformation shall be expressed as a number of foci per dish, the number of dishes with transformed foci, and the number of transformed foci per number of surviving cells.

(2) *Interpretation of results.* (i) There are several criteria for determining a positive result, one of which is a statistically significant concentration-related increase in the number of transformed foci. Another criterion may be based upon the detection of a reproducible and statistically significant positive response for at least one of the test substance concentrations.

(ii) A test substance which does not produce either a statistically significant concentration-related increase in the number of transformed foci or a statistically significant and reproducible positive response at any one of the test points is considered to be negative in this system.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(3) *Test evaluation.* (i) Positive results for an *in vitro* mammalian cell transformation assay indicate that, under the test conditions, the test substance induces morphologic transformation in the cultured mammalian cells used.

(ii) Negative results indicate that, under the test conditions, the test substance does not induce morphologic transformation in the cultured mammalian cells used.

(4) *Test report.* In addition to the reporting recommendations as specified under 40 CFR Part 792 Subpart J, the following specific information shall be reported:

(i) Cell type used, including subclone designation and passage number; number of cell cultures; methods used for maintenance of cell cultures.

(ii) Rationale for selection of concentrations and number of cultures.

(iii) Test conditions: Composition of media, $CO_2$ concentration, concentration of test substance, vehicle, incubation temperature, incubation time, duration of treatment, cell density during treatment, type of metabolic activation system, positive and negative controls, length of expression period (including number of cells seeded and subculture and feeding schedules, if appropriate).

(iv) Methods used to enumerate numbers of viable cells and transformed foci.

(v) Dose-response relationship, where possible.

(g) *References.* For additional background information on this test guideline, the following references should be consulted:

(1) Heidelberger, C., Freeman, A.E., Pienta, R.J., Sivak, A., Bertram, J.S., Castro, B.C., Dunkel, V.C., Francis, M.W., Kakunaga, T., Little, J.B., Schechtman, L.M., "Cell transformation by chemical agents—a review and analysis of the literature: a report of the U.S. Environmental Protection Agency Gene-Tox Program." *Mutation Research* 114:283–385, 1983.

(2) Kakunaga, T. "A quantitative system for assay of malignant transformation by carcinogens using a clone derived from Balb-3T3." *International Journal of Cancer* 12:463–473, 1973.

(3) Reznikoff, C.A., Bertram, J.S., Brankow, D.W., Heidelberger, C. "Quantitative and qualitative studies of chemical transformation of cloned C3H mouse embryo cells sensitive to postconfluence inhibition of cell division." *Cancer Research* 33:3239–3249, 1973.

(4) Reznikoff, C.A., Brankow, D.W., Heidelberger, C. "Establishment and characterization of a cloned line of C3H mouse embryo cells sensitive to postconfluence inhibition of division." *Cancer Research* 33:3231–3238, 1973.

(5) Sivak, A., Charest, M.C., Dudenko, L., Silveira, D.M., Simons, I., Wild, A.W. "Balb/c–3T3 cells as target cells for chemically induced neoplastic transformation." In: *Advances in modern environmental toxicology, mammalian cell transformation by chemical carcinogens,* Vol. I. Mishra, N., Dunkel, V., Mehlman, M., eds. Princeton Junction, NJ: Senate Press, pp. 133–180, 1981.

(6) Sivak, A., Tu, A.S. "Factors influencing neoplastic transformation by chemical carcinogens in Balb/c-3T3 cells." In: *The predictive value of short-term screening tests in carcinogenicity evaluation.* Williams, G.M., Kroes, R., Waaijers, H.W., Van de Poll, K.W., eds. Amsterdam, New York, Oxford: Elsevier/North Holland Biomedical Press, pp. 177–190, 1980.

(7) Williams, G.M. "Detection of chemical carcinogens by unscheduled DNA synthesis in rat liver primary cell culture." *Cancer Research* 37:1845-1851, 1977.

[52 FR 19085, May 20, 1987]

# PART 796—CHEMICAL FATE TESTING GUIDELINES

## Subpart A—[Reserved]

## Subpart B—Physical and Chemical Properties

Sec.
796.1050 Absorption in aqueous solution: Ultraviolet/visible spectra.
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Sec.
796.3800 Gas phase absorption spectra and photolysis.

Authority: 15 U.S.C. 2603.

## Subpart A—[Reserved]

## Subpart B—Physical and Chemical Properties

### § 796.1050 Absorption in aqueous solution: Ultraviolet/visible spectra.

(a) *Introductory information*—(1) *Guidance information*. (i) Molecular formula.

(ii) Structural formula.

(2) *Standard documents*. The spectrophotometric method is based on national standards and consensus methods which are applied to measure the absorption spectra.

(b) *Method*—(1)(i) *Introduction, purpose, scope, relevance, application and limits of test*. (A) The primary environmental purpose in determining the ultraviolet-visible (UV-VIS) absorption spectrum of a chemical compound is to have some indication of the wavelengths at which the compounds may be susceptible to photochemical degradation. Since photochemical degradation is likely to occur in both the atmosphere and the aquatic environment, spectra appropriate to these media will be informative concerning the need for further persistence testing.

(B) Degradation will depend upon the total energy absorbed in specific wavelength regions. Such energy absorption is characterized by both molar absorption coefficient (molar extinction coefficient) and band width. However, the absence of measurable absorption does not preclude the possibility of photodegradation.

(ii) *Definitions and units*. The *UV-VIS absorption spectrum* of a solution is a function of the concentration, $c_i$, expressed in mol/L, of all absorbing species present; the path length, d, of the spectrophotometer cell, expressed in cm; and the molar absorption (extinction) coefficient, $\epsilon_i$, of each species. The absorbance (optical density) A of the solution is then given by:

$$A = d \sum_{i} \epsilon_{\lambda i}$$

For a resolvable absorbance peak, the band width λ is the wavelength range, expressed in nm=$10^{-9}$m, of the peak at half the absorbance maximum.

(iii) *Reference substances.* (A) The reference substances need not be employed in all cases when investigating a new substance. They are provided primarily so that calibration of the method may be performed from time to time and to offer the chance to compare the results when another method is applied.

(B) Reference compounds appropriate for the calibration of the system are:

(*1*) Potassium dichromate (in 0.005 mol/L, $H_2SO_4$ solution) from J.A.A. Ketelaar, paragraph (d)(2) of this section:

| | | | | |
|---|---|---|---|---|
| log ϵ | 3.56 | 3.63 | 3.16 | 3.50 |
| λ in nm | 235 | 257 | 313 | 350 |

(*2*) Fluoranthene (in methanol) from *C.R.C. Atlas of Spectral Data*, paragraph (d)(3) of this section:

| | | | | | |
|---|---|---|---|---|---|
| log ϵ | 4.75 | 4.18 | 4.73 | 3.91 | 3.92 |
| λ in nm | 237 | 236 | 288 | 339 | 357 |

(*3*) 4-nitrophenol (in methanol) from *C.R.C. Atlas of Spectral Data*, paragraph (d)(3) of this section:

| | | |
|---|---|---|
| log ϵ | 3.88 | 4.04 |
| λ in nm | 288 | 311 |

See also paragraph (d)(1) of this section.

(iv) *Principle of the test method.* This method utilizes a double-beam spectrophotometer which records only the absorption differences between the blank and test solutions to give the spectrum of the chemical being tested.

(v) *Quality criteria—Reproducibility and sensitivity.* (A) Reproducibility and sensitivity, need not be measured directly. Instead, the accuracy of the system in measuring the spectra of reference compounds will be defined so as to assure appropriate reproducibility and sensitivity. It is preferable to use a recording double-beam spectrophotometer to obtain the UV-VIS spectrum of the test compound. Such an instrument should have a photometric accuracy of ±0.02 units over the absorbance range of 0 to 2 units. It should be capable of recording absorbances at wavelengths of 200 to 750 nanometers nm with a wavelength accuracy of ±0.5 nm. The cells employed with the instrument must necessarily be transparent over this wavelength range and must have a path length determined to within 1 percent. To ensure that the instrument is performing satisfactorily, spectra for test solutions of $K_2Cr_2O_7$ (for absorbance accuracy) and holmium glass (for wavelength accuracy) should be run periodically.

(B) In the event that a recording double-beam instrument is not available, it will be necessary to determine the absorbance of the test solution in a single-beam instrument at 5-nm intervals over the entire wavelength range and at 1-nm intervals where there are indicated absorbance maxima. Wavelength and absorbance tests should be done as with the double-beam instrument.

(2) *Description of the test procedure*—(i) *Preparation*—(A) *Preparation of test solutions.* (*1*) Solutions should be prepared by accurately weighing an appropriate amount of the purest form of the test substance available. This should be made up in a concentration which will result in at least one absorbance maximum in the range 0.5 to 1.5 units.

(*2*) The absorption of a compound is due to its particular chemical form. It is often the case that different forms are present, depending on whether the medium is acidic, basic, or neutral. Consequently, spectra under all three conditions are required where solubility and concentration allow. Where it is not possible to obtain sufficient concentrations in any of the aqueous media, a suitable organic solvent should be used (methanol preferred).

(*3*) The acid medium should have a pH of less than 2, and the basic medium should be at least pH 10. The solvent for the neutral solution, and for preparing the acidic and basic ones, should be distilled water, transparent to ultraviolet radiation down to 200 nm. If methanol must be used, acidic and basic solutions can be prepared by adding 10 percent by volume of HCl or NaOH in aqueous solution ([HCl], [NaOH] = 1 mol/L).

(*4*) In theory, all chemical species other than that being tested are present in both beams and would therefore not appear in the recorded spectrum of a double-beam instrument. In practice, because the solvent is usually present in great excess, there is a threshold value of wavelength below which it is not possible to record the spectrum of the test chemical. Such a wavelength will be a property of the solvent or of the test medium. In general, distilled water is useful from 200 nm (dissolved ions will often increase this), methanol from 210 nm, hexane from 210 nm, acetonitrile from 215 nm and dichloromethane from 235 nm.

(B) *Blank solutions.* A blank must be prepared which contains the solvent and all chemical species other than the test chemical. The absorption spectrum of this solution should be recorded in a manner identical to that of the test solution and preferably on the same chart. This "baseline" spectrum should never record an absorbance reading varying more than ±0.05 from the nominal zero value.

(C) *Cells.* Cell pathlengths are usually between 0.1 cm and 10 cm. Cell lengths should be selected to permit recording of at least one maximum in the absorbance range of 0.5 to 1.5 units. Which set of cells should be used will be governed by the concentration and the absorbance of the test solution as indicated by the Beer-Lambert Law. The cells should be transparent over the range of the spectrum being recorded, and the path-lengths should be known to an accuracy of at least 1 per cent. Cells should be thoroughly cleaned in an appropriate manner (chromic acid is useful for quartz cells) and rinsed several times with the test or blank solutions.

(ii) *Performance of the test.* Both cells to be employed should be rinsed with the blank solution and then filled with same. The instrument should be set to scan at a rate appropriate for the required wavelength resolution and the spectrum of the blank recorded. The sample cell should then be rinsed and filled with the test solution and the scanning repeated, preferably on the same spectrum chart, to display the baseline. The test should be carried out at 25° C.

(c) *Data and reporting*—(1) *Treatment of results.* (i) The molar absorption coefficient $\epsilon$ should be calculated for all absorbance maxima of the test substance. The formula for this calculation is:

$$\epsilon = \frac{A}{c_M \times d},$$

where the quantities are as defined above (see Definitions and units).

(ii) For each peak which is capable of being resolved, either as recorded or by extrapolated symmetrical peaks, the bandwidth should be recorded.

(2) *Test report.* (i) The report should contain a copy of each of the three spectra (3 pH conditions). If neither water nor methanol solutions are feasible, there will be only one spectrum. Spectra should include a readable wave-length scale. Each spectrum should be clearly marked with the test conditions.

(ii) For each maximum in each spectrum, the $\epsilon$ value and bandwidth (when applicable) should be calculated and reported, along with the wavelength of the maximum. This should be presented in tabular form.

(iii) The various test conditions should be included, such as scan speed, the name and model of the spectrophotom-eter, the slit width (where available), cell type and path length, the concentrations of the test substance, and the nature and acidity of the solvent medium. A recent test spectrum on appropriate reference materials for photometric and wavelength accuracy should also be submitted (see Reproducibility and sensitivity).

(d) *Literature references.* For additional background information on this test guideline, the following references should be consulted:

(1) Milazzo, G., Caroli, S., Palumbo-Doretti, M., Violante, N., *Analytical Chemistry*, 49: 711 (1977).

(2) Katelaar, J.A.A., *Photoelectric Spectrometry Group Bulletin*, 8, (Cambridge, 1955).

(3) Chemical Rubber Company, *Atlas of Spectral Data*, (Cliffland, Ohio).

[50 FR 39472, Sept. 27, 1985]

### § 796.1220 Boiling point/boiling range.

(a) *Introductory information.* (Ebulliometric Method; Dynamic Method; Distillation Method; Siwoloboff Method; Photocell Detection Method).

(1) *Qualifying statement.* The methods and devices described in this test guideline can be applied to liquids, provided that these do not undergo chemical reaction at temperatures below the boiling point (for example: autoxidation, rearrangement, degradation, etc.).

(2) *Additional comments.* The Dynamic Method was tested in the OECD Laboratory Intercomparison Testing Programme, Part I, 1979, for vapor pressure determination.

(3) *Standard documents.* In this guideline, five methods to determine the boiling point are described. The Ebulliometric Method is based on the ASTM D 1120-72. The Distillation Method is based on the standards ISO R 918 and the draft ISO DIS 4626, BS 4359/68, BS 4591/71, DIN 53171. The Siwoloboff Method is based on JIS K 0064-1966. The Photocell Detection Method is based on the manufacturer's manual (4).

(b) *Method*—(1)(i) *Introduction, purpose, scope, relevance, application, and limits of test.* (A)(*1*) The boiling point of a substance is an environmentally relevant physical chemical property because it is important for identification purposes and is one factor influencing the states in which the substance will exist in the environment.

(*2*) The emphasis in this test guideline has been placed on the description of the method using photocell detection, because this method allows the determination of melting as well as boiling points. Moreover, the measurements can be performed automatically.

(B) The Dynamic Method has the advantage that it can also be applied for the determination of vapor pressure and that it is not necessary to correct the boiling temperature to the normal pressure (101.325 kPa) because the standard pressure can be adjusted during the measurement. However, this method is not at present automated. (For a detailed description, see OECD Test Guideline 104 for the Vapor Pressure Curve.)

(C) NOTE: In the literature, different boiling points are sometimes quoted for the same substance. These differences are due to such variables as the dimension of the apparatus (for example, the fit of the thermometer), the type of the thermometer, the stem correction, the pressure correction, and the accuracy of the pressure measurement. Therefore, the above-mentioned international and national standardised methods contain precise requirements for these specified conditions.

(D) The influence of impurities on the determination of the boiling point depends greatly upon the kind of impurity. Thus, the effect can be considered if a highly volatile solvent is present in the sample. Impurities will usually increase/decrease the measured boiling temperature.

(ii) *Definitions and units.* (A) The standard *boiling point* is described as the temperature at which the pressure of the saturated vapor of a liquid is the same as the standard pressure.

(B) The measured boiling point is dependent on the atmospheric pressure. This dependence can be described quantitatively by the Clausius-Clapeyron equation as follows:

$$\log p = -\frac{\Delta H_v}{2.3\,RT} + \text{constant}$$

(where p is the vapor pressure of the substance, Δ $H_v$ is its heat of vaporization, and R is the universal molar gas constant. R=8.31441 J $mol^{-1}K^{-1}$ The temperature T is expressed in K).

(C) The temperature at the boiling point (boiling temperature) is stated in K, with regard to the ambient pressure during the measurement. If no pressure is given, the result refers to a standard pressure of 101.325 kPa.

(D) Conversions:

*Pressure*—(units—kPa)

100 kPa=1 bar=0.1 MPa
("bar" units are still permissible but not recommended)
133 Pa=1 mm Hg=1 Torr
(the units mm Hg and Torr are no longer permissible)

*Temperature*—(units—K)

t=T−273.15
t in °C, and T in K

(*1*) At small deviations from the normal pressure (max. ± 5 kPa), the boiling point temperatures are normalized to $T_n$ by means of the following number-value-equation by Sidney-Young:

$T_n = T + f_T \Delta p$

where:

$\Delta p$=(101.325−p) note sign
p=barometer measurement in kPa
$f_T$=rate of change of boiling point with pressure in K/kPa
T=measured boiling temperature in K
$T_n$=boiling temperature corrected to normal pressure in K

(*2*) The temperature-correction factors $f_T$ and equations for their approximation are included in the international and national standards mentioned in paragraph (b)(2) of this section for many substances. For example, the DIN 53171 method mentions the following rough corrections for solvents included in paints under the following Table 1:

TABLE 1—TEMPERATURE-CORRECTION-FACTORS $F_T$

| Temperature T K | Correction factor $f_T$ K/kPa | Temperature T K | Correction factor $f_T$ K/kPa |
|---|---|---|---|
| 323.15 | 0.26 | 473.15 | 0.39 |
| 348.15 | 0.28 | 498.15 | 0.41 |
| 373.15 | 0.31 | 523.15 | 0.44 |
| 398.15 | 0.33 | 548.15 | 0.45 |
| 423.15 | 0.35 | 573.15 | 0.47 |
| 448.15 | 0.37 | | |

A table of temperature-correction factors for organic solvents (see ISO/DIS 4626) is included in the following Table 2:

TABLE 2—TEMPERATURE-CORRECTION FACTORS FOR ORGANIC SOLVENTS (SEE ISO/DIS 4626)

| Product | Thermometer | Boiling point °C at 101.325 kPa | Rate of change of boiling point with pressure | |
|---|---|---|---|---|
| | | | K' °C/0.1 kPa (°C/mbar) | K °C/mmHg |
| Acetone | 39 C | 56.1 | 0.029 | 0.039 |
| Acetonitrile | 40 C | 81.6 | 0.032 | 0.043 |
| Allyl alcohol | 40 C | 96.9 | 0.026 | 0.036 |
| Allyl chloride | 38 C | 45.1 | 0.029 | 0.039 |
| n-Amyl acetate | 102 C | 149.5 | 0.036 | 0.048 |
| n-Amyl alcohol | 41 C | 138.0 | 0.031 | 0.041 |
| Aromatic solvent naptha | 42 C | | 0.037 | 0.049 |
| Benzene | 40 C | 80.1 | 0.032 | 0.042 |
| iso-Butyl acetate | 41 C | 117.3 | 0.034 | 0.045 |
| n-Butyl acetate | 41 C | 126.1 | 0.034 | 0.045 |
| sec-Butyl acetate | 40 C | 112.4 | 0.034 | 0.045 |
| iso-Butyl-alcohol | 40 C | 107.9 | 0.027 | 0.036 |
| n-Butyl-alcohol | 40 C | 117.7 | 0.028 | 0.037 |
| sec-Butyl-alcohol | 40 C | 99.5 | 0.026 | 0.035 |
| tert-Butyl alcohol | 40 C | 82.5 | 0.025 | 0.033 |
| p-tert-Butyl toluene | 104 C | 192.8 | 0.042 | 0.056 |
| Cumene | 102 C | 152.4 | 0.038 | 0.051 |
| Cyclohexane | 40 C | 80.7 | 0.033 | 0.044 |
| Cyclohexanone | 102 C | 155.7 | 0.038 | 0.051 |
| Diacetone alcohol | 102 C | 169.2 | 0.038 | 0.050 |

TABLE 2—TEMPERATURE-CORRECTION FACTORS FOR ORGANIC SOLVENTS (SEE ISO/DIS 4626)—Continued

| Product | Thermometer | Boiling point °C at 101.325 kPa | Rate of change of boiling point with pressure | |
|---|---|---|---|---|
| | | | K' °C/0.1 kPa (°C/mbar) | K °C/mmHg |
| Di-iso-butyl ketone | 103 C | 169.3 | 0.036 | 0.051 |
| Di-iso-butylene | 40 C | 101.4 | 0.034 | 0.046 |
| 1,2-Dichloroethane | 40 C | 83.5 | 0.032 | 0.043 |
| Dichloromethane | 38 C | 39.8 | 0.028 | 0.037 |
| Diethyleneglycol | 106 C | 245.8 | 0.038 | 0.050 |
| Diethylene glycol-mono-n-butyl ether | 105 C | 230.4 | 0.038 | 0.051 |
| Diethylene glycol-monoethyl ether | 104 C | 201.9 | 0.036 | 0.048 |
| Diethylene glycol-monomethyl ether | 104 C | 193.8 | 0.035 | 0.047 |
| Dimethyl formamide | 102 C | 153.0 | 0.033 | 0.044 |
| Dipropylene glycol | 106 C | 232.8 | 0.038 | 0.051 |
| Di-iso-propyl ether | 39 C | 68.3 | 0.031 | 0.041 |
| Ethyl acetate | 39 C | 77.2 | 0.031 | 0.041 |
| Ethyl alcohol | 39 C | 78.3 | 0.025 | 0.033 |
| Ethyl benzene | 41 C | 136.2 | 0.037 | 0.049 |
| Ethylene glycol | 104 C | 197.6 | 0.032 | 0.043 |
| Ethylene glycol-mono-n-butyl ether | 103 C | 171.2 | 0.035 | 0.047 |
| Ethylene glycol-monoethyl ether | 102 C | 135.1 | 0.033 | 0.044 |
| Ethylene glycol-monoethyl ether acetate | 102 C | 156.3 | 0.035 | 0.046 |
| Ethylene glycol-monomethyl ether | 41 C | 124.5 | 0.031 | 0.041 |
| Ethylene glycol-mono-iso-propyl ether | 102 C | 142.8 | 0.033 | 0.044 |
| 2-Ethyl hexanol | 104 C | 184.8 | 0.034 | 0.046 |
| Ethyl-iso-amyl ketone | 103 C | 158.2 | 0.037 | 0.049 |
| n-Hexyl acetate | 103 C | 171.6 | 0.038 | 0.050 |
| Hexylene glycol | 104 C | 197.1 | 0.034 | 0.045 |
| Isophorone | 105 C | 215.3 | 0.043 | 0.057 |
| Mesityloxide | 41 C | 129.8 | 0.035 | 0.047 |

TABLE 2—TEMPERATURE-CORRECTION FACTORS FOR ORGANIC SOLVENTS (SEE ISO/DIS 4626)—Continued

| Product | Thermometer | Boiling point °C at 101.325 kPa | Rate of change of boiling point with pressure | |
|---|---|---|---|---|
| | | | K' °C/0.1 kPa (°C/mbar) | K °C/mmHg |
| 4-Metoxy-4-methyl-2-pentanone | 103 C | 160.6 | 0.037 | 0.049 |
| Methanol | 39 C | 64.6 | 0.025 | 0.033 |
| Methyl-iso-amyl acetate | 102 C | 146.2 | 0.036 | 0.048 |
| Methyl-iso-amyl ketone | 102 C | 144.9 | 0.036 | 0.048 |
| Methyl-iso-butyl carbinol | 41 C | 131.8 | 0.031 | 0.041 |
| Methyl-iso-butyl ketone | 41 C | 116.2 | 0.035 | 0.046 |
| Mono-ethanol amine | 103 C | 170.7 | 0.030 | 0.040 |
| Perchloroethylene | 41 C | 121.2 | 0.036 | 0.048 |
| n-Propyl acetate | 40 C | 101.6 | 0.032 | 0.042 |
| iso-Propyl acetate | 40 C | 88.5 | 0.031 | 0.041 |
| n-Propyl alcohol | 40 C | 97.2 | 0.026 | 0.034 |
| iso-Propyl alcohol | 40 C | 82.3 | 0.025 | 0.033 |
| Propylene glycol | 104 C | 187.6 | 0.032 | 0.043 |
| Propylene oxide | 38 C | 34.3 | 0.027 | 0.036 |
| Pyridine | 41 C | 115.4 | 0.035 | 0.046 |
| Toluene | 41 C | 110.6 | 0.035 | 0.046 |
| Triethylene glycol | 107 C | 287.6 | 0.038 | 0.050 |
| Triethylene glycol-monoethyl ether | 106 C | 255.4 | 0.038 | 0.051 |
| Trichloroethylene | 40 C | 87.1 | 0.032 | 0.043 |
| Vinyl acetate | 39 C | 72.7 | 0.030 | 0.040 |
| White spirit | 103 C | | 0.041 | 0.055 |
| Xylene (isomer mixture) | 41 C | | 0.037 | 0.049 |
| m-Xylene | 41 C | 139.1 | 0.037 | 0.049 |
| o-Xylene | 41 C | 144.4 | 0.037 | 0.050 |
| p-Xylene | 41 C | 138.3 | 0.037 | 0.049 |

(iii) *Reference substances.* The standard methods listed include specifications for calibration and evaluation substances. These compounds need not be employed in all cases when investigating a new substance. They should primarily serve to calibrate the method from time to time and to offer the chance to compare the results when another method is applied.

(iv) *Principle of the test methods.* All methods for the determination of the boiling point (boiling range) are based on the measurement of the boiling temperature.

(A) *Determination by use of the Ebulliometer.* See paragraphs (d) (1) and (4) of this section. Ebulliometers were originally developed for the determination of molecular weight by boiling point elevation, but they are also suited for exact boiling point measurements. A very simple apparatus is described in ASTM D 1120-72. The liquid is heated in this apparatus under equilibrium conditions at atmospheric pressure until it boils. The determined temperature of the liquid, corrected to standard pressure, is the boiling point.

(B) *Dynamic method.* See paragraph (d)(2) of this section. This method measures the vapor recondensation temperature by means of a thermocouple in the reflux while boiling. The pressure can be varied in this method.

(C) *Distillation method for boiling point (and boiling range).* This method involves distillation of the liquid and measurement of the vapor recondensation temperature and determination of the amount of distillate.

(D) *Method according to Siwoloboff.* See paragraph (d)(2) of this section. A sample is heated in a sample tube which is immersed in a heat-bath liquid. A fused capillary, containing an air bubble in the lower part, is dipped in the sample tube. The temperature at which a regular string of bubbles escapes from the capillary or the temperature at which the string of bubbles stops and the fluid suddenly starts rising in the capillary (Siwoloboff under paragraph (d)(2) of this section) is determined.

(E) *Photocell detection.* See paragraph (d)(3) of this section. Using the principle according to Siwoloboff. Measurements are automatic, the rising bubbles being detected photoelectrically.

(v) *Quality criteria.* The different methods for the determination of the boiling point (boiling range) are compared with regard to their use and precision and possibility to standardize/automate in the following Table 3:

TABLE 3—COMPARISON OF THE METHODS

| Method of measurement | Approximate accuracy | Ability to standardise | Automation |
|---|---|---|---|
| Ebulliometer.... | ±1.4 K (up to 373 K) [1].<br>±2.5 K (above 373 K) [1]. | Existing standard ASTM D 1120-72 [1]. | Difficult as yet. |
| Dynamic method. | ±0.5 K.......... | Possible.......... | Difficult. |
| Distillation process (Boiling range). | ±0.5 K.......... | Existing standards, e.g., ISO/R 918, DIN 53171, BS 4591/17. | Difficult as yet. |
| According to Siwoloboff. | ±1K to ±2K.. | Possible.......... | Automatic method exists see: photocell detection. |
| Photocell detection. | ±0.3 K (at 373 K). | Possible.......... | Measurement process works automatically |

[1] This accuracy is only valid for pure substances and for the simple device as, for example, described in ASTM D 1120-72; it can be improved with more sophisticated ebulliometer devices.

(2) *Description of the test procedures.* The procedures of several of the test methods have been established by various international and national standards mentioned above. Reference is made here to those standards which prescribe details of preparations, test conditions, and conduct of the test.

(i) *Ebulliometer.* See: ASTM D 1120-72, Standard Test Method for Boiling Point of Engine Antifreezes, and reference under paragraph (d)(4) of this section.

(ii) *Dynamic method.* See: OECD Test Guideline 104 for Vapour Pressure Curve, Principle of the test methods. The boiling temperature observed with an applied pressure of 101.325 kPa is recorded.

(iii) *Distillation process (boiling range).*

See: ISO/R 918, Test Method for Distillation (Distillation Yield and Distillation Range),

ISO 4626/1980, Volatile Organic Liquids—Determination of Boiling Range of Organic Solvents Used as Raw Materials, BS 4349/68, Method for Determination of Distillation of Petroleum Products,

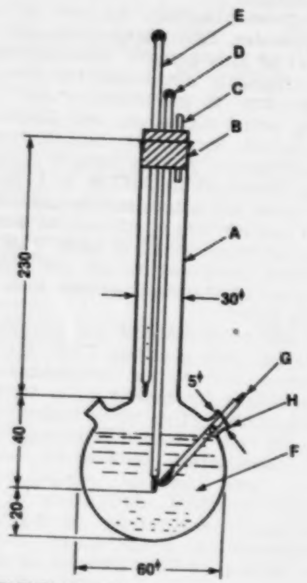
BS 4591/71, Method for the Determination of Distillation Characteristics,

DIN 53171, Lösungsmittel für Anstrichstoffe, Bestimmung des Siedeverlaufes,

DIN 51751, Prüfung flüssiger Mineralkohlenwasserstoffe—Bestimmung des Siedeverlaufes.

(iv) *Method according to Siwoloboff.* (A) The sample is heated in a melting point apparatus in a sample tube, with a diameter of approximately 5 mm in the following Figure 1:

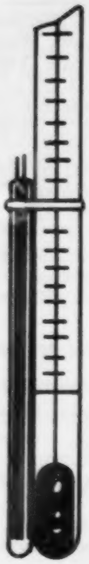
FIGURE 1—APPARATUS FOR THE DETERMINATION OF MELTING AND BOILIN POINT (JISK 0064-1966), WITH SPECIFICATIONS IN MM



A: MEASURING TUBE
B: CORK STOPPER
C: VENT
D: THERMOMETER
E: AUXILIARY THERMOMETER
F: BATH LIQUID
G: SAMPLE TUBE; MAX. 5mm OUTER DIAMETER; CAPILLARY TUBE, APPROX. 1mm INNER DIAMETER, AND APPROX. 0.2mm TO 0.3mm WALL-THICKNESS
H: SIDE TUBE

(B) A capillary tube (boiling capillary) fused about 1 cm above the lower end is placed in the sample tube. The level to which the test substance is filled is such that the fused section of the capillary is below the surface of the liquid. The sample tube containing the boiling capillary is fastened either to the thermometer with a rubberband or is fixed with a support from the side under the following Figure 2:

FIGURE 2—PRINCIPLE ACCORDING TO SIWOLOBOFF

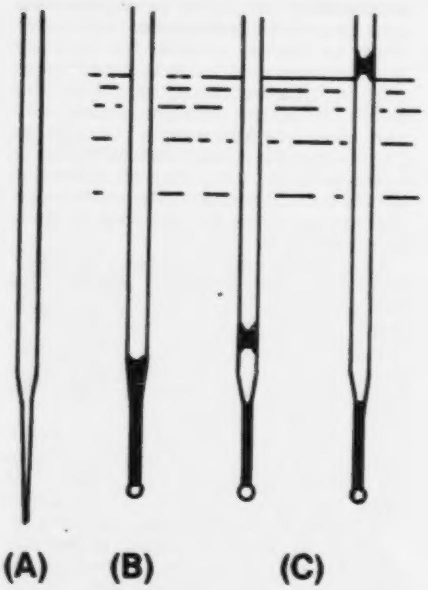


(C) The bath liquid is chosen according to boiling temperature. At temperatures of up to 573 K sulfuric acid or silicon oil can be used. Liquid paraffin may only be used up to 473 K. The heating of the bath liquid should be adjusted to a temperature rise of 3 K/min at first. The bath liquid must be stirred. At about 10 K below the expected boiling point, the heating is reduced so that the rate of temperature rise is less than 1 K/min. When the boiling temperature is approached, bubbles begin to emerge from the boiling capillary.

(D) The boiling point is reached when the string of bubbles stops and fluid suddenly starts rising in the capillary. The corresponding thermometer reading is the boiling temperature of the substance.

(E) In the modified principle, under the following Figure 3, the boiling point is determined in the melting point capillary which is stretched to a fine point about 2 cm in length (1) and a small amount of the sample is aspirated. The open end of the fine capillary is closed by melting, so that a small air bubble is located at the end. When heated in the melting point apparatus (2), the air bubble expands. The boiling point corresponds to the temperature at which the substance plug reaches the level of the surface of the bath liquid (3).

FIGURE 3—MODIFIED PRINCIPLE



(v) *Photocell detection.* (A) The sample is heated in a capillary tube inside a heated metal block. A light beam is directed via suitable holes in the block through the substance onto a precisely calibrated photocell. During the increase of the sample temperature, single air bubbles emerge from the boiling capillary. When the boiling temperature is reached, the amount of bubbles increases immensely.

(B) This causes a change in the intensity of light, which is recorded by a photocell and gives a stop signal to the digital indicator reading out the temperature of a platinum resistance thermometer, located in the block.

(C) This method is especially useful because it allows determinations below room temperature as low as 253.15 K (−20°C) without any changes in the apparatus. The instrument merely has to be placed in a cold room or cooling bath. The exact execution of the boiling point determination can be obtained from the instrument manual.

(3) *General remarks.* (i) The results obtained for mixtures or impure samples are to be interpreted with care. With an impure sample, for instance, the emergence of a low boiling component will be registered as the boiling point. Repeated determinations with the same impure sample can change the composition from measurement to measurement, due to the volatilization of low boiling components: continously increasing values are obtained in these circumstances.

(ii) Liquids with a tendency to superheat can yield incorrect results. The values obtained are usually too high. This happens more frequently at higher temperatures. Distillation methods or the dynamic vapor pressure method are more suitable for these types of compound.

(c) *Data and reporting*—(1) *Treatment of results.* (i) The boiling point to be determined should be a mean of at least two measurements, which are in the range of approximate accuracy indicated in Table 2 under paragraph (b)(1)(v) of this section. If determinations are not reproducible, other methods should be considered (see General remarks, above).

(ii) The measured boiling points and their mean should be stated in K, and the pressure(s) at which the measurement(s) was (were) made should be recorded in kPa. Where a test substance boils over a temperature range, this range should be provided. The measured values should also be corrected to standard pressure. Estimates of accuracy should be provided for all results.

(iii) The method used should be indicated, including any deviations from procedures described in this Test Guideline.

(2) [Reserved]

(d) *Literature references.* For additional background information on this test guideline the following references should be consulted:

(1) Klenitz, H. *Methoden der Organischen Chemie* ed. Houben-Weyl, Vol. 2, (Georg Thieme Verlag, Stuttgart, 1953, pp. 815–821.

(2) *Test Guideline 104 for Vapour Pressure Curve,* A 80/5 Umweltbundesamt, Berlin (1980).

(3) Siwoloboff, A. *Berichte-Deutsche Chemische Gesellschaft* 19: 795 (1886).

(4) Manual of Apparatus FP 5+FP 51, FP 52 and FP 53, Mettler Instrumente AG, CH-8606 Greifensee-Zürich, Switzerland.

(5) *European Pharmacopoeia,* 1:75 (1974).

[50 FR 39473, Sept. 27, 1985]

## § 796.1370 Dissociation constants in water.

(a) *Introductory information.* (Titration Method; Spectrophotometric Method; Conductometric Method)

(1) *Prerequisites.* (i) Suitable analytical method.

(ii) Water solubility.

(2) *Guidance information.* (i) Structural formula.

(ii) Electrical conductivity for conductometric method.

(3) *Qualifying statements.* (i) All test methods may be carried out on pure or commercial grade substances. The possible effects of impurities on results should be considered.

(ii) The *titration method* is not suitable for low solubility compounds (see Test solutions, under paragraph (b)(2)(ii)(A) of this section.

(iii) The *spectrophotometric method* is only applicable to compounds having appreciably different UV/VIS absorption spectra for the dissociated and undissociated forms. This method may also be suitable for low solubility compounds and for non-acid/base dissociations, e.g. complex formation.

(iv) In cases where the Onsager equation holds, the *conductometric method* may be used, even at moderately low concentrations and even in cases for non-acid/base equilibria.

(4) *Standard documents.* This Test Guideline is based on methods given in the references listed in section 4 and on the Preliminary Draft Guidance

for Premanufacture Notification EPA, August 18, 1978.

(b) *Method*—(1) *Introduction, Purpose, Scope, Relevance, Application and Limits of Test.* (i) The dissociation of a chemical in water is of importance in assessing its impact upon the environment. It governs the form of the substance which in turn determines its behavior and transport. It may affect the adsorption of the chemical on soils and sediments and adsorption into biological cells.

(ii) *Definitions and units. Dissociation* is the reversible splitting into two or more chemical species which may be ionic. The process is indicated generally by

$$RX \rightleftharpoons R^{+} + X^{-}$$

and the concentration equilibrium constant governing the reaction is

$$K = \frac{[R^{+}][X^{-}]}{[RX]}$$

For example, in the particular case where R is hydrogen (the substance is an acid), the constant is

$$K_a = [H^{+}] \cdot \frac{[X^{-}]}{[HX]}$$

or

$$pK_a = pH - \log \frac{[X^{-}]}{[HX]}$$

(iii) *Reference substances.* (A) The following reference compounds need not be employed in all cases when investigating a new substance. They are provided primarily so that calibration of the method may be performed from time to time and to offer the chance to compare the results when another method is applied.

|  | $pK_a$ (1) | Temp. in °C |
|---|---|---|
| p-Nitrophenol | 7.15 | 25[1] |
| Benzoic acid | 4.12 | 20 |
| p-Chloroaniline | 3.93 | 20 |

[1] No value for 20 °C is available, but it can be assumed that the variability of measurement results is higher than the temperature dependence to be expected.

(B) It would be useful to have a substance with several pK's as indicated in Principle of the method, under paragraph (b)(1)(iii) of this section. Such a compound could be:

|  | $pK_a$ (8) | Temp. in °C |
|---|---|---|
| Citric acid | (1) 3.14 | 20 |
|  | (2) 4.77 | 20 |
|  | (3) 6.39 | 20 |

(iv) *Principle of the test method.* The chemical process described is generally only slightly temperature dependent in the environmentally relevant temperature range. The determination of the dissociation constant requires a measure of the concentrations of the dissociated and undissociated forms of the chemical substance. From a knowledge of the stoichiometry of the dissociation reaction indicated in Definitions and units, above, the appropriate constant can be determined. In the particular case described in this guideline the substance is behaving as an acid or a base, and the determination is most conveniently done by determining the relative concentrations of ionized and un-ionized forms of the substance and the pH of the solution. The relationship between these terms is given in the equation for $pK_a$ in Definitions and units, above. Some compounds exhibit more than one dissociation constant and similar equations can be developed. Some of the methods described herein are also suitable for non-acid/base dissociation.

(v) *Quality criteria*—(A) *Repeatability.* The dissociation constant should be replicated (a minimum of three determinations) to within ±0.1 log units.

(B) [Reserved]

(2) *Description of the test procedures.* (i) There are two basic approaches to the determination of $pK_a$.

One involves titrating a known amount of substance with standard acid or base, as appropriate; the other involves determining the relative concentrations of the ionized and un-ionized forms and their pH dependence.

(ii) *Preparations.* Methods based on those principles may be classified as titration, spectrophotometric and conductometric procedures.

(A) *Test solutions.* (*1*) For the titration method and the conductometric method the chemical substance should be dissolved in distilled water. For spectrophotometric and other methods buffer solutions are used. The concentrations of the test substances should not exceed the lesser of 0.01 M or half the saturation concentration, and the purest available form of the substance should be employed in making up the solutions. If the substance is only sparingly soluble, it may be dissolved in a small amount of a water-miscible solvent prior to adding to the concentrations indicated above.

(*2*) Solutions should be checked for the presence of emulsions using a Tyndall beam, especially if a co-solvent has been used to enhance solubility. Where buffer solutions are used, the buffer concentration should not exceed 0.05 M.

(B) [Reserved]

(iii) *Test conditions*—(A) *Temperature.* (*1*) The temperature should be controlled to at least ±1 °C. The determination should preferably be carried out at 20 °C.

(*2*) If a significant temperature dependence is suspected, the determination should be carried out at at least two other temperatures. The temperature intervals should be 10 °C in this case and the temperature control ±0.1 °C.

(B) *Analyses.* The method will be determined by the nature of the substance being tested. It must be sufficiently sensitive to allow determination of the different species at the test solution concentrations.

(iv) *Performance of the test.* (A) *Titration method.* The test solution is determined by titration with the standard base or acid solution as appropriate, measuring the pH after each addition of titrant. At least 10 incremental additions should be made before the equivalence point. If equilibrium is reached sufficiently rapidly, a recording potentiometer may be used. For this method both the total quantity of substance and its concentration need to be accurately known. Precautions must be taken to exclude carbon dioxide. Details of procedure, precautions, and calculation are given in standard tests, e.g., references under paragraphs (d) (1), (2), and (4) of this section.

(B) *Spectrophotometric methods.* A wavelength is found where the ionized and un-ionized forms of the compound have appreciably different extinction coefficients. The UV/VIS absorption spectrum is obtained from solutions of constant concentration under a pH condition where the substance is essentially un-ionized and fully ionized and at several intermediate pH's. This may be done either by adding increments of concentrated acid (base) to a relatively large volume of a solution of the compound in a multicomponent buffer, initially at high (low) pH under paragraph (d)(5) of this section, or by adding equal volumes of a stock solution of the compound in, e.g., water, methanol, to constant volumes of various buffer solutions covering the desired pH range. From the pH and absorbance values at the chosen wavelength, a sufficient number of values for the $pK_a$ is calculated using data from at least 5 pH's where the compound is at least 10 percent and less than 90 percent ionized. Further experimental details and method of calculation are given in reference under paragraph (d)(1) of this section.

(C) *Conductometric method.* Using a cell of small, known cell constant, the conductivity of an approximately 0.1 M solution of the compound in conductivity water is measured. The conductivities of a number of accurately made dilutions of this solution are also measured. (The concentration is halved each time, and the series should cover at least an order of magnitude in concentration.) The limiting conductivity at infinite dilution is found by carrying out a similar experiment with the Na salt and extrapolating. The degree of dissociation may then be calculated from the conductivity of each solution using the Onsager equation, and hence using the Ostwald Dilution Law the dissociation constant may be calculated as

$K=\alpha_2 C/1-\alpha$

where C is the concentration in moles per litre and $\alpha$ is the fraction dissociated.

Precautions must be taken to exclude $CO_2$. Further experimental details and method of calculation are given in standard texts and references under paragraphs (d) (1) and (7) of this section.

(c) *Data and reporting*—(1) *Treatment of results*—(i) *Titration method.* The $pK_a$ is calculated for 10 measured points on the titration curve. The mean and standard deviation of such $pK_a$ values are calculated. A plot of pH versus volume of standard base or acid should be included along with a tabular presentation.

(ii) *Spectrophotometric methods.* The absorbance and pH are tabulated from each spectrum. At least five values for the $pK_a$ are calculated from the intermediate spectra data points, and the mean and standard deviation of these results are also calculated.

(iii) *Conductometric method.* The equivalent conductance, $\Lambda$, is calculated for each acid concentration and for each concentration of a mixture of one equivalent of acid, plus 0.98 equivalent of carbonate-free sodium hydroxide. (The excess acid is to prevent an excess of $OH^-$ due to hydrolysis.) $1/\Lambda$ is plotted against $\sqrt{C}$ and $\Lambda_o$ of the salt can be found by extrapolation to zero concentration. $\Lambda_o$ of the acid can be calculated using literature values for $H^+$ and $Na^+$. The $pK_a$ can be calculated from $\alpha=\Lambda_i/\Lambda_o$ and $Ka=\alpha^2C/1-\alpha$ for each concentration. Better values for Ka can be obtained by making corrections for mobility and activity. The mean and standard deviations of the $pK_a$ values should be calculated.

(2) *Test report.* (i) All raw data and calculated $pK_a$ values should be submitted together with method of calculation (preferably in a tabulated format, such as suggested in paragraph (d)(1) of this section) as should the statistical parameters described under paragraph (c)(1) of this section. For titration methods, details of the standardization of titrants should be given.

(ii) For spectrophotometric methods, all spectra should be submitted. For the conductometric method, details of the cell constant determination should be reported. Information on technique used, analytical methods and the nature of any buffers used should be given.

(iii) The test temperature(s) should be reported.

(d) *Literature references.* For additional background information on this test guideline the following references should be consulted:

(1) Albert, A. & Sergeant, E.P., *Ionization Constants of Acids and Bases.* (Wiley, Inc., New York, 1962).

(2) Nelson, N.H. & Faust, S.D., "Acidic Dissociation Constants of Selected Aquatic Herbicides," *Environment Science Technology* 3: II (1969).

(3) ASTM D 1293. American Society for Testing and Materials, 1916 Race St., Philadelphia, PA 19103.

(4) Standard Method 242. *Standard Methods for the Examination of Water and Waste Water.* 14th ed., (American Public Health Association: Washington, DC, 1976).

(5) Clark, J. & Cunliffe, A.E., "Rapid Spectrophotometric Measurement of Ionisation Constants in Aqueous Solution," Chem. Ind. (London 1973).

(6) ASTM D 1125. American Society for Testing and Materials, 1916 Race St. Philadelphia, PA 19103.

(7) Standard Method 205. *Standard Methods for the Examination of Water and Waste Water.* 14th ed. (American Public Health Association: Washington, DC, 1976).

(8) *Handbook of Chemistry and Physics,* 60th ed. (CRC-Press: Boca Raton, Florida, 1980).

[50 FR 39477, Sept. 27, 1985]

## § 796.1520 Particle size distribution/fiber length and diameter distributions.

(a) *Introductory information.* Method A: Particle Size Distribution (effective hydrodynamic radius). Method B: Fiber Length and Diameter Distributions.

(1) *Prerequisites.* Method A: Water insolubility. Method B: Information on fibrous nature of product; Information on stability of fiber shape under electron-microscopic conditions.

(2) *Guidance information.* Method A: Melting point. Method B: Melting point.

(3) *Qualifying statements.* Both test methods can be applied to pure and commercial grade substances.

(i) *Method A:* (*A*) This method can only be applied to water-insoluble ($<10^{-6}$ g/l), powdered type products.

(*B*) The equivalence of the six national and international standard methods for particle size distribution was not tested, and is currently not known. There is a particular problem in relation to sedimentation and Coulter counter measurements.

(ii) *Method B:* This method applies only for fibrous products. The effect of impurities on particle shape should be considered.

(4) *Recommendations.* Method A: Equivalence of the methods for determination of particle size distribution should be tested in the laboratory.

(5) *Standard documents.* The "Effective Hydrodynamic Radius Determination" is based on the following standards:

(i) ASTM—D 3360, D422.
(ii) NF-T 30044.
(iii) DIN—66115.
(iv) DIN—66116, Part 1.
(v) ASTM—C 678.
(vi) ANSI—C 690-75.

and on a test principle described in *Chem. Ing. Tech.* 46: 729 (1974).

(b) *Method*—(1) *Introduction, purpose, scope, relevance, application and limits of test.* (i)(A) Many methods are available for particle size measurements, but none of them is applicable to the entire size range. Sieving, microscopic sedimentation and elutriation techniques are most commonly employed. Moreover, in the case of airborne particles (dusts, smokes, fumes), radiation scattering and inertial methods prove particularly useful. Finally, appropriate sampling procedures should be selected in order to prepare specimens really representative of the material under test (Method A).

(B) The first method described in this guideline (Method A) is designed to provide information on the transportation and sedimentation of insoluble particles in water and air. In the special case of materials which can form fibers, an additional set of measurements (Method B) is also recommended to help identify potential health hazards arising from inhalation or ingestion.

(C) Method A is generally applicable, frequent in use and hydrodynamic in character; Method B is comparatively specialized, infrequently required and involves microscopic examination. It should be borne in mind, however, that the original particle size distribution is highly dependent on the industrial processing methods used and can also be affected by subsequent environmental or human transformations.

(D) These tests are applicable only to water insoluble (solubility $<10^{-6}$ g/l) substances. Method B for fibers will be applied only if light microscopic examination, similarities to known fibrous or fiber-releasing substances or other data indicate a likelihood that fibers are present. In this context, a fiber is a water insoluble particle, of aspect ratio (length/diameter) >3 and diameter <100 µm. Fibers of length <5 µm need not be considered. Method A, which determines the effective hydrodynamic radius, $R_s$, will be used for both fibrous and non-fibrous particulates without prior inspection. It is useful only in the range 2 µm$<R_s<$100 µm.

(ii) *Definitions and units.* (A) For Method A the parameter of interest is the effective hydrodynamic radius, or effective Stokes radius $R_s$. The terminal velocity of a small sphere falling under the influence of gravity in a viscous fluid is given by:

$$v=2_g R^2_s(d_1-d_2)/9\eta$$

Where

$v$=velocity (m/sec),
$g$=gravitation constant (m/sec$^2$),
$R_s$=Stokes radius (m)
$d_1$=density of sphere (kg/m$^3$),
$d_2$=density of fluid (kg/m$^3$),
$\eta$=dynamic viscosity (Nsec/m$^2$=Pa s) of the the fluid

(B) In other situations, similar relationships apply. Particle size is usually measured in micrometers ($=10^{-6}$ m).

(C) Method B provides histograms of the length (l) and diameter (d) distributions of fibres. The ordinate is the absolute number of particles in each interval of 1 or d. Typical plots are provided in Figures 1 and 2 under paragraph (b)(1)(iv) of this section.

(iii) *Reference substances.* (A) Five reference substances of defined particle size covering the overall range 0.35 to 650 µm (excepting the 50 to 200 µm region) have been certified with respect to the cumulative mass distribution of particles versus equivalent settling rate diameter or equivalent volume diameter. The materials will be made available from the Community Bureau of Reference of the European Economic Community and they will be issued with certificates of measurement. The certification report under paragraph (d) (4) of this section will also be available from the Community Bureau of Reference.

(*1*) The certification report of five reference materials will be available from: Commission of the European Community, Directorate—General for Research, Science and Education, Community Bureau of Reference BCR, rue de la Loi 200, B-1049 Brussels.

(*2*) Filter equipment for sample preparations according to Method B is available commercially through the following manufacturers:

(*i*) Nuclepore Corporation, 7035 Commerce Circle, Pleasanton California 94566/USA.

(*ii*) Millipore Corporation, Order Service Department, Bedford, Massachusetts 01730/USA.

(*iii*) Whatman Filters, W&R Balston Limited, England.

(B) *Calibration materials*—(*1*) *Method A.* A binary or ternary mixture of latex spheres (2 µm <d <100 µm) is suggested.

(*2*) *Method B.* No standard reference materials are readily available.

(C) *Evaluation materials*—(*1*) *Method A.* A ternary mixture of 2 µm, 50 µm and 100 µm latex spheres (which provides a discrete calibrated distribution) plus a sample of crushed quartz (continuous distribution).

(*2*) *Method B.* Fibrous chrysotile asbestos is recommended (specific properties not essential as long as enough of a throughly mixed sample is available for identical distribution in a ring test).

(iv) *Principle of the test methods*—(A) *Method A.* (*1*) There are several standard methods available which meet the sensitivity requirements:

| Principle | Methods |
|---|---|
| Sedimentation | ASTM—D 3360, D 422, NF-T 30044 DIN—66-115 |
| Centrifugation | ASTM—C 678, Chem.Ing.Tech. 46, 729 (1974) |
| Coulter counter | ANSI-C 690-75. |

(*2*) The comparability of these methods (especially the sedimentation) and the other methods must be determined.

(*3*) The sample should also be subjected to a simple light microscopic examination to determine the approximate nature of the particles (e.g, plates, needles, etc.).

(B) *Method B*—Since data must be collected on small diameter fibers (>0.1 µm), scanning (SEM) or transmission (TEM) electron microscopy is required. There is no standard procedure at present, and those currently under development for asbestos contamination (in which the fibrous material is already identified and in high concentration) are often more complex and expensive than necessary for the needs of this program. Extreme care must still be taken during sample preparation to avoid fiber breaking, clumping and contamination. A simple initial procedure is suggested below (Description of the test procedures). The length and diameter of the fiber images can be measured manually, semi-automatically or automatically and the results tabulated in histogram form (see the following Figures 1 and 2):

FIGURE 1—SAMPLE FIBER LENGTH DISTRIBUTION (METHOD B)

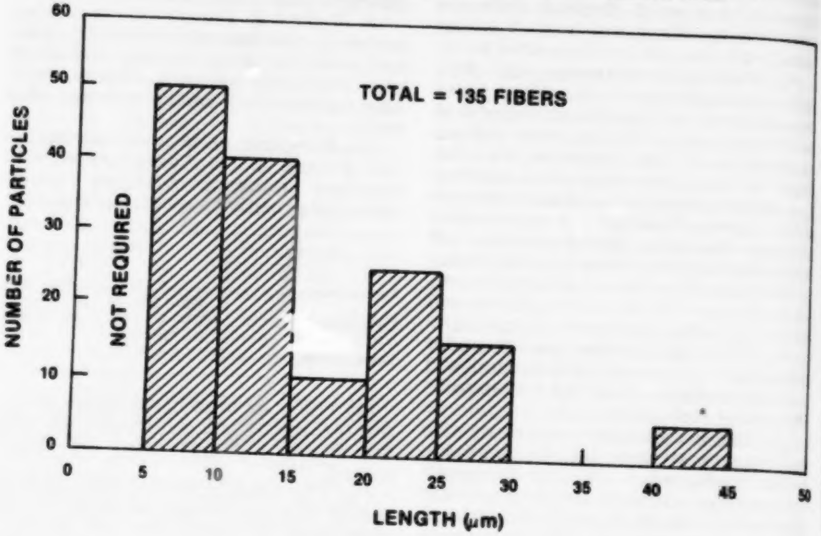
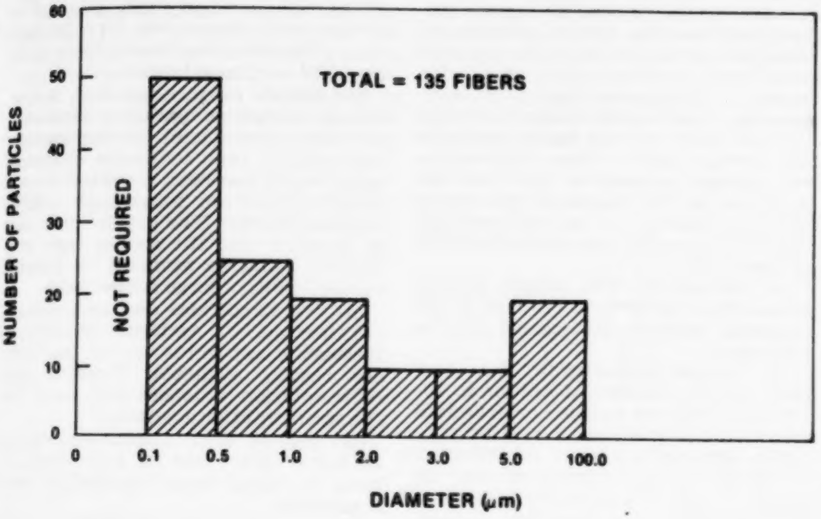


FIGURE 2—SAMPLE FIBER DIAMETER DISTRIBUTION (METHOD B)



(v) *Quality criteria*—(A) *Repeatability.* (*1*) The effective hydrodynamic radius distribution (Method A) should be measured three times, with no two values differing by more than 20 percent.

(*2*) The length and diameter distributions of fibers (Method B), if required, should be measured at least twice—using separate samplings and preparations—with at least 70 fibers *per* histogram. No two values in a given histogram interval should differ by more than 50 percent or 3 fibers, whichever is larger. Such repeatability should be sufficient for the modeling and decision-making procedures currently envisaged; however, the presence of long, thin fibers—due to their potential adverse health effects—would indicate a need for further, more precise measurements.

(B) *Sensitivity.* In the general case (Method A) particles as small as 2 μm and as large as 200 μm must be measurable. The method requires that sufficient numbers of radius intervals be used to resolve the radius distribution curve. In the case of fibers (Method B), diameters as small as 0.2 μm and as large as 100 μm and lengths as small as 5 μm and as large as 300 μm, must be measurable.

(C) *Specificity.* See paragraph (b)(1) of this section.

(D) *Possibility of standardisation.* The method *procedures* can be readily standardized, if desired, but non-uniformity of sampling, preparation and prior handling may still cause considerable variation in results in Method B.

(E) *Possibility of automation.* Automation or semi-automation of these procedures if possible. Full automation of fiber 1 and d measurements and analysis is also possible.

(2) *Description of the test procedures*—(i) *Preparations*—(A) *Method A.* The small quantities used as samples must be representative of product batches comprising many kilograms; therefore, sampling and sample handling require great care. For example, small particles often form agglomerates; therefore, sample pre-treatment

(e.g., the addition of dispersing agents, agitation, or low-level ultrasonic treatment) may be required before the primary particle size can be determined. However, great care must be taken to avoid changing the particle size distribution. In the case of highly stable aggregates, a strict distinction between primary particles and agglomerates is not always useful. Some representative sample preparation methods will be found in the standard procedures listed in Principle of the test methods (Method A) under paragraph (b)(1)(iv) of this section.

(B) *Method B.* Two simple sample preparation procedures (B-1, B-2) for scanning electron microscopy can be suggested.

(*1*) *Sample preparation B-1.* Suspend a given amount of sample in 10-100 ml of filtered distilled or deionized water (the suspension should be relatively light, not a slurry). Distribution of the particles in suspension may be aided by use of a surfactant, such as small amounts (~1 part/100) of absolute ethyl alcohol or a non-ionic detergent. Suspension of the powder is achieved by gentle hand agitation, vortex mixing or magnetic stirring. Filter the suspension directly onto a 47 mm diameter Nuclepore* filter overlaying a 47 mm diameter Millipore* membrane filter housed in a 47 mm diameter Millipore* filter holder (Hydrosol, stainless) using gentle vacuum. Ensure that the powder has not precipitated out of suspension. Depending on the size of particles of interest various pore-sized filters may be used. The concentration of suspended particles determines the amount filtered. A less concentrated suspension will give a more even distribution of particles on the filter surface under paragraph (d)(2) of this section. Remove the Nuclepore filter from the filter housing, being careful not to disturb the particles on the surface. Place the filter—particle-coated face upward—into a glass or plastic Petri dish containing Whatmann No. 1 filter paper; cover Petri dish and store in a dry box or under vacuum. When completely dried, the filter is cut into pieces of appropriate size and mounted, filter face up, onto copper tape which has been previously mounted onto an SEM specimen holder (using double face tape). To ensure stickiness of the tape, preheat using infrared or similar heat source for 5 to 15 minutes. Trim the edge of the filter to fit the SEM specimen holder.

(*2*) *Sample preparation B-2.* An alternate sample preparation method is the direct transfer of the dry powder onto copper tape (adhesive electrical tape) which has been mounted onto a scanning electron microscope (SEM) specimen holder. The powder may also be sprayed onto the copper tape surface by using an atomizer or pipette equipped with a large rubber bulb.

(ii) *Test conditions and apparatus*—(A) *Method A.* Ambient conditions. Measuring apparatus for all methods are readily available. Pipettes and sedimentation balances are used for the sedimentation methods.

(B) *Method B.* (*1*) Contamination by air-borne fibers can be a problem. A hood or "clean room" should be used if available.

(*2*) A small electron microscope and support equipment are required.

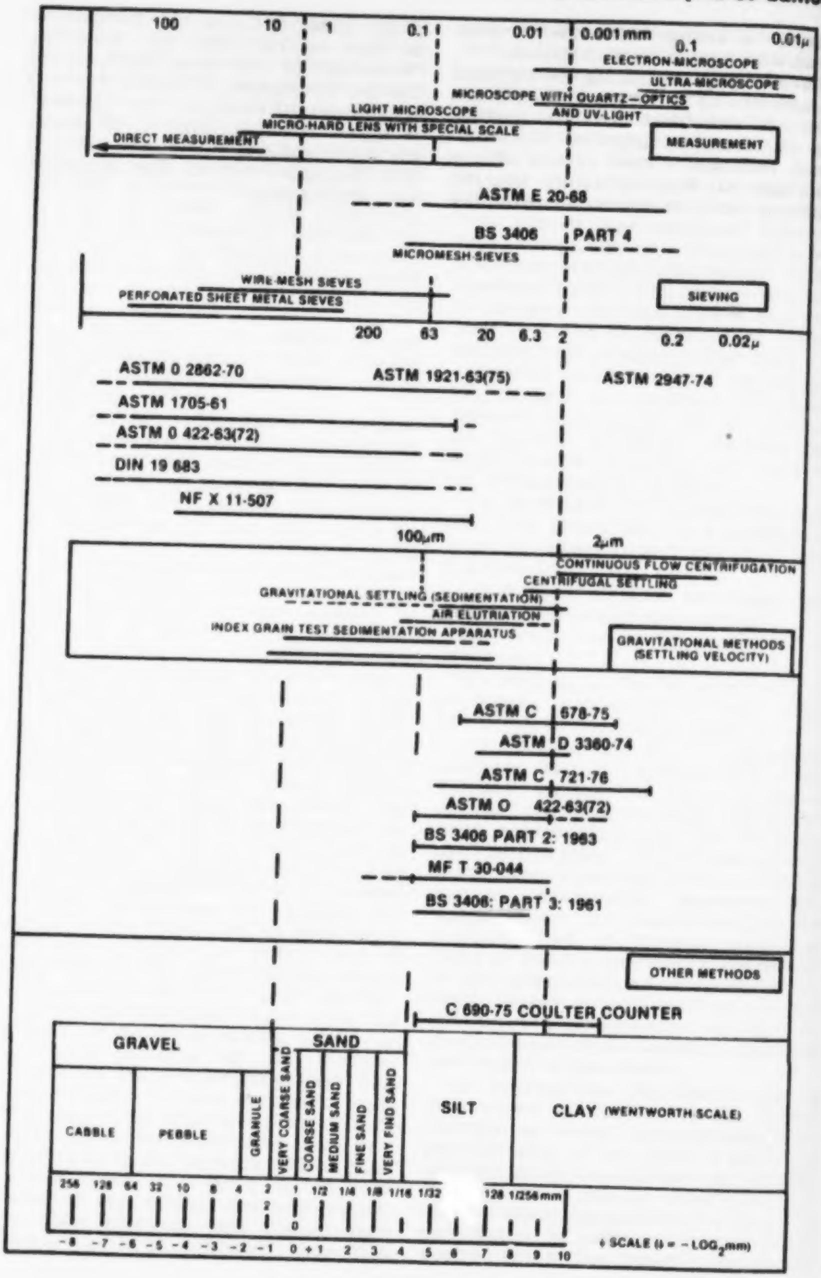
(iii) *Performance of the tests.* (A) methods:

(*1*) *Method A.* To be selected from standard procedures listed above (Principle of the test methods).

(*2*) *Method B.* Both preparation methods (B-1 and B-2) provide a particulate sample on filter paper or copper tape mounted on an SEM specimen holder. This can then be examined in the SEM, or first coated with metal film using a sputtering device or vacuum evaporator. Representative fields within the sample surface are photographed at various magnifications to yield a representative sample of the population of interest. (If desired, energy dispersive x ray analysis (EDXA) of representative particles—to check sample contamination—could be performed at this time.)

(B) Particle size distribution can be determined by measuring the screen directly or from measurements on photographs. If the SEM is equipped with an image analysis system, population statistics can be determined directly. Such measurements can be automated or semi-automated when desired (3). If the image indicates the sample is too concentrated, repeat again with a more dilute solution.

(iv) *Analysis.* Measuring the physical parameters by different methods can result in somewhat different particle size distributions; therefore, the measuring techniques used should always be reported. Representative analysis methods are discussed in reference under paragraphs (d) (1) through (6) of this section, and the following "Summary of the Usual Methods for the Determination of Particle Size and the Important Granular Size Classes," (adapted from G. Müller, *Methoden der Sedimentuntersuchungen*, 1964, p. 303, Stuttgart, revised with appropriate supplements):


100
10
1
0.1
0.01
0.001 mm
0.1
0.01μ
ELECTRON-MICROSCOPE
ULTRA-MICROSCOPE
MICROSCOPE WITH QUARTZ—OPTICS AND UV-LIGHT
LIGHT MICROSCOPE
MICRO-HARD LENS WITH SPECIAL SCALE
DIRECT MEASUREMENT
MEASUREMENT
ASTM E 20-68
BS 3406 PART 4
MICROMESH SIEVES
WIRE-MESH SIEVES
PERFORATED SHEET METAL SIEVES
SIEVING
200
63
20
6.3
2
0.2
0.02μ
ASTM 0 2862-70
ASTM 1921-63(75)
ASTM 2947-74
ASTM 1705-61
ASTM 0 422-63(72)
DIN 19 683
NF X 11-507
100μm
2μm
CONTINUOUS FLOW CENTRIFUGATION
CENTRIFUGAL SETTLING
GRAVITATIONAL SETTLING (SEDIMENTATION)
AIR ELUTRIATION
INDEX GRAIN TEST SEDIMENTATION APPARATUS
GRAVITATIONAL METHODS (SETTLING VELOCITY)
ASTM C 678-75
ASTM D 3360-74
ASTM C 721-76
ASTM O 422-63(72)
BS 3406 PART 2: 1963
MF T 30-044
BS 3406: PART 3: 1961
OTHER METHODS
C 690-75 COULTER COUNTER
GRAVEL
SAND
CABBLE
PEBBLE
GRANULE
VERY COARSE SAND
COARSE SAND
MEDIUM SAND
FINE SAND
VERY FIND SAND
SILT
CLAY (WENTWORTH SCALE)
256
128
64
32
16
8
4
2
1
1/2
1/4
1/8
1/16
1/32
1/128
1/256 mm
-8
-7
-6
-5
-4
-3
-2
-1
0
+1
2
3
4
5
6
7
8
9
10
φ SCALE (φ = − LOG$_2$mm)

(c) *Data and reporting*—(1) Data. (i) Method A: Data should be obtained for 3 size ranges: >200 μm, <2 μm and the region 2 to 200 μm. Only in the latter range should the distribution curve be prepared. It should have sufficient μm increments to resolve the curve (subpopulations). A histogram presentation is required plus a statement on the weight percent of material >200 μm and <2 μm.

(ii) Method B: Full length (l) and diameter (d) data are needed on fibers of dimensions d>0.1 μm and l>5 μm. Two histogram distributions, based on examination of at least 50 fibers each, should be prepared. For diameters, the ranges should be 0.1-0.5, 0.5-1.0, 1-2, 2-3, 3-5 μm and over 5 μm. For lengths they should be 0-5, 5-10, 10-15, 15-20, (etc.) μm. This is illustrated in Figures 1 and 2, above.

(2) *Test report.* (i) Method A: The following information should be presented:

(A) Expected percent change of reported values in the future (e.g., variations between production batches).

(B) Sample preparation methods used.

(C) Analysis methods used.

(D) Approximate information on particle shape (e.g., spherical, platelike, needle shaped).

(E) Lot number, sample number.

(F) Suspending medium, temperature, pH.

(G) Concentration.

(H) Stoke's (effective hydrodynamic) radius $R_s$ distribution for $2<R_s<200$ μm.

(I) Mean value and approximate "area" (percent) of any resolvable peaks in $R_s$ distribution.

(J) Percent of particles with $R_s<2$ μm.

(K) Percent of particles with $R_s>200$ μm.

(ii) Method B: The following information should be presented:

(A) Sample description, method description.

(B) Number of particles *per* field.

(C) Total number of fibers measured.

(D) l, d distributions (histograms).

(E) Mean value and approximate "area" (percent) of any resolvable peaks in the $R_s$ distribution.

(d) *Literature references.* For additional background information on this test guideline the following references should be consulted:

(1) Allen, T., *Particle Size Measurement.* (Chapman and Hall: London, 1975).

(2) Irani, R.R., and Callis, C.F., *Particle Size Measurement.* Interpretation and Application.

(3) Orr, S., and Dallavalle, J.M., *Fine Particle Measurement.*

(4) *Certification Report on Particles of Defined Particle Size.* (Community Bureau of Reference: Brussels, 1979).

(5) McGrath, P.P., and Ewell, J.B., "Application of Electron Microscopy to Problem of Particulate Contaminants in Food, Drugs and Biologicals," *Scanning Electron Microscopy,* Part III (1976).

(6) *Symposium on Electron Microscopy of Microfibers,* ed. Asher I.M., and McGrath, P.P., Proceedings of the First FDA Office of Science Summer Symposium, (August 23-25, 1976).

[50 FR 39479, Sept. 27, 1985]

### § 796.1550 Partition coefficient (n-Octanol/water).

(a) *Introduction*—(1) *Background and purpose.* (i) Bioconcentration, the accumulation of a substance in living tissues or other organic matter as a result of net chemical uptake from the medium (e.g., water), is a factor in determining the movement of a chemical in the environment and the potential effects of the chemical on biota. Hydrophobic chemicals that are present in the aqueous environment at subtoxic concentrations may accumulate to toxic levels once inside organisms, presumably through diffusion into nonpolar cell components, where they accumulate because of their greater solubility. Further movement of the substance in living tissues may occur as a result of ingestion of lower trophic level organisms, i.e., food chain effects.

(ii) The tendency of an organic chemical to bioconcentrate in living cells can be inferred from the value of the octanol/water partition coefficient, $K_{ow}$ (Neely et al. 1974) under paragraph (d)(5) of this section. Chemicals with $K_{ow}$ less than 10 will not significantly partition into, or tend to accumulate in, living cells. Chemicals with $K_{ow}$ greater than $10^6$ will tend to accumulate. Chemicals that exist in the environment at subtoxic levels may bioconcentrate to toxic levels once inside organisms.

(iii) This test guideline describes a detailed and commonly used procedure for determining the octanol/water partition coefficient of organic chemicals.

(2) *Definitions and units.* (i) The octanol/water partition coefficient ($K_{ow}$) is defined as the equilibrium ratio of the molar concentrations of a chemical in n-octanol and water, in dilute solution. $K_{ow}$ is a constant for a given chemical at a given temperature. Since $K_{ow}$ is the ratio of two molar concentrations, it is a dimensionless quantity. Sometimes $K_{ow}$ is reported as log 10 $K_{ow}$. The mathematical statement of $K_{ow}$ is:

*Equation 1*

$$K_{ow} = \frac{C_{octanol}}{C_{water}} ,$$

where C is the molar concentration of the solute in n-octanol and water at equilibrium at a given temperature.

(ii) The distribution law applies only to individual molecular species in solution. If a molecule dissociates or associates in octanol and water, than equation 1 under paragraph (a)(2)(i) of this section must be modified. In general, if $\alpha$ represents the fraction of the total solute that is dissociated or associated, assuming that either association or dissociation occurs in each solvent, then

*Equation 2*

$$K_{ow} = \frac{(1-\alpha_{oct.})\, C_{oct.}}{(1-\alpha_{water})\, C_{water}} ,$$

since $(1-\alpha)$ gives the fraction of unchanged molecules in each phase. For the special case where no association takes place in octanol, equation 2 reduces to

*Equation 3*

$$K_{ow} = \frac{C_{oct.}}{(1-\alpha_{water})\, C_{water}} ,$$

where $\alpha$ water represents the fraction of the total solute that has dissociated in water.

(3) *Principle of the test method.* The conventional method for determining the octanol/water partition coefficient is carried out by distributing a chemical between n-octanol and water in a vessel at constant temperature and measuring the concentration in the two liquid phases after equilibration (e.g., Fujita et al. 1964 under paragraph (d)(2) of this section; Hansch and Anderson 1967 under paragraph (d)(3) of this section; Leo et al. 1971 under paragraph (d)(4) of this section; Chiou et al. 1977 under paragraph (d)(1) of this section).

(4) *Applicability and specificity.* The test guideline is designed to determine the octanol/water partition coefficient of solid or liquid organic chemicals in the range 10 to $10^6$. For chemicals whose values lie outside this range, $K_{ow}$ should be characterized as less than 10 or greater than $10^6$ with no further quantification.

(b) *Test procedures*—(1) *Test conditions*—(i) *Special laboratory equipment.* (A) A thermostatic bath, chamber, or room with a shaker and temperature control as specified in Temperature Control below.

(B) An ultracentrifuge with temperature control as specified in Temperature Control below.

(C) Stainless steel or glass centrifuge tubes with sealable caps. Special glass centrifuge tubes can be used up to approximately 12,000 G and stainless steel tubes can be used at high G values.

(D) A mechanical shaker.

(E) A pH meter capable of resolving differences of 0.1 pH unit or less.

(ii) *Temperature control.* The temperature of the water bath, or chamber, or room, and the ultracentrifuge shall be controlled to (25±1) °C.

(iii) *Solvents.* N-octanol, purified as described in paragraph (b)(2)(i)(A) of this section, and distilled or reagent grade water, i.e., ASTM Type II water or an equivalent grade, shall be used. ASTM Type II water is described in ASTM D 1193-77 (Reapproved 1983), "Standard Specification for Reagent Water." ASTM D 1193-77 (Reapproved 1983) is available for inspection at the Office of the Federal Register, Rm. 8301, 1100 L St., NW., Washington, DC. This incorporation by reference was approved by the Director of the Office of the Federal Register. This material is incorporated as it exists on the date of approval and a notice of any change in this material will be published in the FEDERAL REGISTER. Copies of the incorporated material may be obtained from the TSCA Public Docket Office (TS-793), Rm NE-G004, Office of Toxic Substances, Environmental Protection Agency, 401 M St., SW., Washington, DC 20460, and from the American Society for Testing and Materials (ASTM), 1916 Race Street, Philadelphia, PA 19103.

(iv) *Concentration of solute.* All experiments shall be carried out at solute concentration $C<0.01M$ (Molar) in octanol and water and well below the solubility in either phase.

(v) *Equilibration time.* In general, 1 hour of gentle agitation is sufficient to reach equilibrium. For surfactants, at least 16 hours is required to reach equilibrium.

(vi) *Octanol/water volume ratio.* The ratio of the volumes of the two liquids shall be adjusted as appropriate for the relative solubility of the chemical in octanol and water. By adjusting the volumes, concentration errors (resulting from analytical errors) are minimized and errors resulting from dividing large numbers by small numbers are kept to a minimum.

(vii) *Chemical analysis of the octanol and water phases.* In determining the $K_{ow}$ value for any given solute, both the octanol and water phases shall be analyzed unless analysis of the octanol phase indicates a $\log_{10} K_{ow}$ >6 for the chemical. An analytical method shall be selected that is most applicable to the analysis of the specific chemical. Chromatographic methods are preferable because of their compound specificity in analyzing the parent chemical without interference from impurities. Whenever practicable, the chosen analytical method shall have a precision with ±5 percent.

(viii) *Emulsion and ultracentrifugation.* Gentle shaking shall be used to minimize the formation of emulsions. Ultracentrifugation is necessary to separate troublesome emulsions and to separate the octanol and water phases unless turbidometric measurements are used to demonstrate that the emulsion is broken. Ultracentrifugation shall be carried out at 25 °C for 20 minutes in a temperature-controlled ultracentrifuge. The acceleration (G) value required to break the emulsion and to achieve complete separation of the octanol and water phases shall be determined by trial-and-error experimentation.

(ix) *Equilibration vessel.* (A) If feasible, equilibration should be carried out in a centrifuge tube (stainless steel or glass) with a sealable cap. The centrifuge tubes shall be almost completely full. In this way, partitioning with air will be minimized, especially for volatile chemicals, and the mixture will be completely mixed.

(B) Very hydrophobic chemicals, with $K_{ow}$ in the order of $10^4$ to $10^8$, require relatively large volumes of the aqueous phase. Hence, for these chemicals, it is recommended that equilibration be carried out in a large ground-glass stoppered flask.

(x) *Speciation effects.* (A) The octanol/water partition coefficient, $K_{ow}$, has been defined in paragraph (a)(2) of this section. The mathematical statement of $K_{ow}$ is given by equation 1 under paragraph (a)(2)(i) of this section.

(B) If the chemical does not associate or dissociate in octanol and water, then use equation 1 under paragraph (a)(2)(i) of this section and determine $K_{ow}$ at molar concentrations $C<0.01M$ and $C_1=0.01C$.

(C) If the chemical associates in octanol or water or in both liquids, then use equation 1 under paragraph (a)(2)(i) of this section and determine $K_{ow}$ at molar concentrations $C<0.01M$, $C_1=0.1C$, $C_2=0.01C$, $C_3=0.001C$ * * * . When $K_{ow}$ is constant at two molar concentrations differing by a factor of

10, then the effect of association has been minimized or eliminated.

(D) If a molecule dissociates or associates in octanol and water, then equation 1 under paragraph (a)(2)(i) of this section shall be modified to take into account such speciation changes as ionization, aggregation, and hydration. For the special case where no association takes place in water, equation 3 under paragraph (a)(2)(ii) of this section shall be used. For chemicals that reversibly ionize or protonate (e.g., carboxylic acids, phenols, or anilines), use equation 3 under paragraph (a)(2)(ii) of this section with water buffered at pH 5.0, 7.0, and 9.0. Buffers described in paragraph (b)(2)(i)(B) of this section shall be used.

(xi) *Prerinsing of all transfer vessels.* All transfer vessels shall be prerinsed with a portion of the equilibrium phase prior to transfer for analysis. This is especially important for very hydrophobic chemicals.

(2) *Preparations*—(i) *Reagents and Solutions*—(A) *Octanol and Water.* Very pure *n*-octanol shall be obtained as follows: wash pure *n*-octanol (minimum 98 percent pure) sequentially with 0.1N $H_2SO_4$, with 0.1N NaOH, then with distilled water until neutral. Dry the *n*-octanol with magnesium sulfate and distill twice in a good distillation column under reduced pressure [b.p. about 80 °C at 0.27 kPa (2 torr)]. The octanol produced shall be at least 99.9 percent pure. Alternatively, a grade equivalent to Fisher Scientific Co. No. A-402 "Certified Octanol-1" shall be used. Distilled or reagent grade (ASTM Type II) water shall be used.

(B) *Buffer solutions.* Prepare buffer solutions using reagent grade chemicals in distilled or reagent grade water as follows:

pH 5.0—To 250 mL of 0.1M potassium hydrogen phthalate add 113 mL of 0.1M sodium hydroxide; adjust final volume to 500 mL with reagent grade water.

pH 7.0—To 250 mL of 0.1 potassium dihydrogen phosphate add 145 mL of 0.1M sodium hydroxide; adjust final volume to 500 mL with reagent grade water.

pH 9.0—To 250 mL of 0.07M borax add 69 mL of 0.1M HCl; adjust final volume to 500 mL with reagent grade water.

Check the pH of each buffer solution at 25 °C with a pH meter and adjust to pH 5.0, 7.0, or 9.0, if necessary.

(C) *Presaturation of the solvents.* Before a partitioning experiment is carried out, prepare octanol saturated with water and water saturated with octanol. Add purified *n*-octanol to a large stock bottle and sufficient distilled water to saturate it. Shake the flask for 24 hours on a mechanical shaker. Then allow sufficient time for the mixture to stand so that the two phases separate. Repeat this procedure using another large stock bottle containing distilled water and sufficient octanol to saturate it. The desired quantities of the presaturated solvents shall be taken from these stock bottles for each partition experiment.

(D) *Preparation of test solution.* Prepare a $10^{-2}$ to $10^{-3}$ M solution of the test material in octanol.

(3) *Performance of the test.* (i) Add a small volume of the octanol test solution (1 to 5 mL) to a centrifuge tube with a sealable cap as described in paragraph (b)(1)(ix) of this section.

(ii) Add the required volume of water to the centrifuge tube as described in paragraph (b)(1)(vi) of this section. The volume of water required is variable, depending upon the amount of chemical required for the analysis. Generally, 20-40 mL of water should be sufficient. Make sure that the centrifuge tube is almost completely full. In this way, partitioning with air will be minimized. This is important, especially when determining $K_{ow}$ for volatile chemicals.

(iii) Equilibrate the samples at 25 °C in constant temperature bath, chamber, or room by gently shaking the centrifuge tube for 1 hour. Avoid vigorous shaking that may cause troublesome emulsions to form. For surfactants, a minimum of 16 hours of shaking is required as described in paragraph (b)(1)(v) of this section.

(iv) Centrifuge the samples at 25 °C for 20 minutes to break any emulsion and to separate the octanol and water phases. Evidence for breaking the emulsion and separation of the water and octanol phases can be obtained using a turbidimeter. The acceleration (G) value required to break the emulsion and to achieve complete separation of the octanol and water phases shall be determined by trial-and-error experimentation.

(v) Sample the octanol and water phases as follows:

(A) Withdraw by pipet a known volume of the octanol phase (approximately ½ or less of the total octanol phase) and transfer to an analysis cell or diluting solvent. Before transferring the aliquot of the octanol phase, wipe the outside of the pipet with a paper tissue.

(B) Remove by pipet the remainder of the octanol phase including the interfacial layer and discard.

(C) Insert another clean pipet close to the bottom of the centrifuge tube and carefully withdraw a known volume of the aqueous phase. Wipe the bottom exterior part of the pipet with a tissue and discharge the aqueous sample directly into an analysis cell or extraction solvent. Do not allow the extraction solvent to contact the pipet stem.

(vi) Select an analytical method that is most applicable to the analysis of the specific chemical as described in paragraph (b)(1)(vii) of this section. Determine the concentration in the octanol and water phases. Express the concentration of the chemical in octanol and water in moles/liter (M).

(vii) Determine the partition coefficient in triplicate (steps i through vii) at two concentrations of the test material $C<0.01M$ and $C_1=0.1C$ as described in paragraph (b)(1)(x) of this section. If $K_{ow}$ is not constant at C and $C_1$, then association effects shall be considered. Therefore, follow steps 1 through 7 at lower concentrations until $K_{ow}$ is constant at two concentrations differing by a factor of 10 as described in paragraph (b)(1)(x) of this section.

(viii) Very hydrophobic chemicals (with $K_{ow}$ on the order of $10^4$ to $10^8$) requires relatively large volumes of the aqueous phase as described in paragraphs (b)(1) (vi) and (ix) of this section. Hence, for very hydrophobic materials, equilibrate the octanol and water phases in a large ground-glass stoppered flask as described above in step (iii). For the final phase separation, transfer the two phase mixture to centrifuge tubes that have been prerinsed with some of the aqueous phase; centrifuge as described in step (iv); withdraw aliquots from each centrifuge tube as described in step (v); and recombine for analysis.

NOTE: Prerinse all transfer tubes with the water phase. Complete steps (vi) and (vii) to determine $K_{ow}$.

(ix) For materials that reversibly ionize or protonate, determine $K_{ow}$ at pH 5.0, 7.0, and 9.0 as described in paragraph (b)(1)(x) of this section. Follow the steps in paragraph (b)(1) (i) through (vii) of this section using the buffered aqueous solutions described in paragraph (b)(2)(i)(B) of this section. Using the acid dissociation constant and the concentration of the chemical in the aqueous phase $[C_{water}]$, the term $\alpha$ can be calculated. The concentration of undissociated chemical can be determined from $\alpha$ and $C_{water}$.

(c) *Data and reporting*— (1) *Test report*. For each individual determination, report the octanol/water partition coefficient at each concentration of the test substance, including the molar concentration of chemical in each phase $[C_{oct}$ and $C_{water}]$. In addition, report the mean value of $K_{ow}$, and the standard deviation.

(2) *Specific analytical and recovery procedures*. (i) Provide a detailed description or reference for the analytical procedure used, including the calibration data and precision; and

(ii) If extraction methods were used to separate the solute from the octanol and aqueous phases, provide a description of the extraction data.

(3) *Other test conditions*. Report the experimental (G) value required to break the emulsion and to achieve separation of the octanol and water phases.

(d) *References*. For additional background information on this test guideline the following references should be consulted:

(1) Chiou, C.T., Freed, V.H., Schmedding, D.W., Kohnert, R.L. "Partition Coefficient and Bioaccumulation of Selected Organic Chemicals," *Environmental Science and Technology*, 11:475 (1977).

(2) Fujita, T., Iwasa, J., Hansch, C. "A New Substituent Constant Derived from Partition Coefficients," *American Chemical Society Journal*, 86:5175 (1964).

(3) Hansch, C., Anderson, S.M., "The Effect of Intermolecular Hydrophobic Bonding on Partition Coefficients," *Journal of Organic Chemistry*, 23:2583 (1977).

(4) Leo, A., Hansch, C., Elkins, D. "Partition Coefficients and Their Uses," *Chemical Reviews*, 71:525 (1971).

(5) Neely, W.B., Branson, D.R., Blau, G.E. "Partition Coefficient to Measure Bioconcentration Potential of Organic Chemicals in Fish," *Environmental Science and Technology*, 8:113 (1974).

[50 FR 39252, Sept. 27, 1985, as amended at 52 FR 19057, May 20, 1987; 52 FR 26150, July 13, 1987; 53 FR 10391, Mar. 31, 1988; 53 FR 12525, Apr. 15, 1988]

### § 796.1570 Partition coefficient (n-Octanol/water)—Estimation by liquid chromatography.

(a) *Introduction*—(1) *Background and purpose.* (i) Since the pioneering work of Hansch and Fujita (1964) under paragraph (d)(1) of this section in the measurement and estimation of the octanol/water partition coefficient ($K_{ow}$), this property has become the cornerstone of a myriad of structure-activity relationships (SAR). Hansch and Leo (1979) under paragraph (d)(2) of this section have used the coefficient extensively for correlating structural changes in drugs with changes observed in biological, biochemical or toxic effects. These correlations are then used to predict the effect of a new drug for which a $K_{ow}$ could be measured.

(ii) In the study of the environmental fate of organic chemicals, the coefficient has become a key parameter. It has been shown to be correlated to water solubility, soil/sediment adsorption coefficient, and bioconcentration. The importance of this property to SAR is indicated by its discussion in the first chapter of Lyman, Reehl and Rosenblatt's (1982) under paragraph (d)(3) of this section comprehensive compendium of methods for estimating the behavior of organic compounds in the environment. These authors consider the measurement or estimation of the coefficient to be the necessary first step in assessing the fate of new chemicals.

(iii) Of the three properties that can be estimated from $K_{ow}$, water solubility is the most important because it affects both the fate and transport of chemicals. For example, highly soluble chemicals become quickly distributed by the hydrologic cycle, have low adsorption coefficients for soils and sediments, and tend to be more easily degraded by microorganisms. In addition, chemical transformation processes such as hydrolysis and oxidation tend to occur more readily if a compound is soluble.

(iv) Direct correlations between $K_{ow}$ and both the soil/sediment adsorption coefficient and the bioconcentration factor are to be expected. In these cases compounds that are more soluble in octanol (more hydrophobic) would be expected to partition out of the water and onto the organic portion of soils/sediments and into lipophilic tissue. The relationship between $K_{ow}$ and the bioconcentration factor, as developed by Neely et al. (1974) under paragraph (d)(4) of this section, and other similar relationships, are the principal means of estimating bioconcentration factors. These factors are then used to predict the potential for a chemical to accumulate in living tissue. As a rough estimate, chemicals with $K_{ow}$ less than 10 will not accumulate in tissue while those with $K_{ow}$ greater than $10^6$ will. Thus, although a chemical may be present in the aqueous environment at subtoxic concentrations, if its $K_{ow}$ is greater than $10^6$ it would accumulate to levels that may be toxic not only to the organism but also to the consumers of that organism.

(v) This test guideline describes a rapid, inexpensive method based on reverse phase-high pressure liquid chromatography (RP-HPLC) for estimating the octanol/water partition coefficient as developed by Veith et al. (1979) under paragraph (d)(5) of this section. It is not intended, however, to replace the standard shake-flask method described in § 796.1550, and should be used keeping in mind the limitations described herein. The RP-

HPLC method is intended to give quick estimates of $K_{ow}$, particularly for very hydrophobic substances and mixtures that cannot be analyzed using the shake-flask method.

(2) *Definitions.* (i) The octanol/water partition coefficient ($K_{ow}$), as estimated by this test method, is the ratio of the equilibrium molar concentrations of a chemical in n-octanol and water, in dilute solution; as such it is a dimensionless quantity. $K_{ow}$ is a constant for a given chemical at a given temperature. Because $K_{ow}$ can assume such a wide range of values, from less than one to greater than a million, depending on the structure of the compound, $K_{ow}$ is often reported as log $K_{ow}$.

(ii) The retention time, $t_R$, is the time in minutes elapsed between sample injection into the chromatograph and the peak maximum (concentration) as recorded on a chromatogram. The retention time is characteristic of the substance, the liquid phase flow rate, and the stationary phase, at a given temperature. With proper flow and temperature control, it can be reproduced to within one percent and used to identify multiple peaks. Although several substances can have nearly identical retention times, each substance has only one retention time. This retention time is not influenced by the presence of other components. Retention times for this method vary between several minutes for substances with a lower $K_{ow}$ to thirty minutes or greater for substances with higher $K_{ow}$'s.

(3) *Principle of the test method.* This test method is based on a reverse-phase high pressure chromatographic (HPLC) separation procedure developed by Veith et al. (1979) under paragraph (d)(5) of this section. The test substance (solute) is injected onto an HPLC column containing a support onto which a long-chain hydrocarbon has been permanently bonded. A methanol/water solvent system is used to elute the solute which is subsequently analyzed using an ultraviolet absorption detector, gas chromatograph, liquid scintillator or other suitable detector. During elution, the solute moves alone the column by partitioning between the mobile phase and the stationary hydrocarbon phase. The retention time on the column is a function of the hydrophobicity of the solute: A water soluble solute has a short retention time while a hydrophobic solute has a long retention time. Once the retention time is measured on the chromatogram, the $K_{ow}$ of the substance is estimated from a previously established linear regression equation between log $t_R$ and log $K_{ow}$. The relationship between these two variables is determined through a calibration step that involves injecting into the chromatograph a mixture of six reference chemicals having a range of retention times and known octanol/water partition coefficients. The retention time for each chemical is measured and a plot of log $t_R$ *vs.* log $K_{ow}$ is made. The data are also correlated using a linear regression and the resulting equation is used to calculate log $K_{ow}$ from the log $t_R$ of test substances; the correlation coefficient of the linear regression gives a measure of the "goodness of fit" of the calibration data to a straight line.

(4) *Applicability and specificity.* The test method described in this guideline is designed to calculate an estimated value of the octanol/water partition coefficient using an empirically derived equation that relates the $K_{ow}$ of a substance to its experimentally determined retention time on a HPLC column. It must be emphasized that the shake-flask method in § 796.1550 remains the conventional method for determining $K_{ow}$. The HPLC method described herein is a rapid procedure for estimating log $K_{ow}$ for a single substance or a mixture of substances. Estimates of log $K_{ow}$ should be limited to within two log units of the minimum and maximum values of the calibration substances, i.e., the method is applicable to substances with log $K_{ow}$ between zero and eight. In the range of two to six log units, estimates are within 22.5±20.1 percent of the values reported in the literature obtained using other methods (Veith et al. 1979) under paragraph (d)(5) of this section.

(b) *Test procedures*—(1) *Test conditions*—(i) *Special laboratory equipment.* (A) A liquid chromatograph equipped with a 6000 psi pump, a high-pressure stopflow injector, and appropriate recorder.

(B) A preparative scale reverse phase column (250 mm × 8 mm under paragraph (a)(4) of this section), e.g., Varian Preparative Micropak C-H, consisting of a stainless steel tube filled with 10 micron LiChrosorb to which octadecylsilane is permanently bonded.

(C) For chemicals that absorb in the ultraviolet (i.e., aromatics), either 254-nm fixed wavelength detector or 190 to 600 nm variable wavelength detector, can be used. For chemicals that cannot be detected in the ultraviolet, a fraction collector can be used to collect fractions at suitable intervals (0.50 to 1.0-minutes near the retention time) for analysis by gas chromatography, liquid scintillation, or other suitable, sensitive, analytical detector.

(ii) *Purity of solvents and reagents.* All solvents (water, methanol, acetone, and cyclohexane) and reagents used in this test procedure should be reagent or HPLC grade and contain no impurities that could interfere with the determination of the retention time of the test compound. Water meeting ASTM Type II standards or an equivalent grade is recommended to minimize the effects of dissolved salts and other impurities. ASTM Type II water is described in ASTM D 1193-77 (Reapproved 1983), "Standard Specification for Reagent Water". ASTM D 1193-77 (Reapproved 1983) is available for inspection at the Office of the Federal Register, Rm. 8301, 1100 L St., NW., Washington, DC. This incorporation by reference was approved by the Director of the Office of the Federal Register. This material is incorporated as it exists on the date of approval and a notice of any change in this material will be published in the FEDERAL REGISTER. Copies of the incorporated material may be obtained from the TSCA Public Docket Office (TS-793), Rm. NE.-G004, Office of Toxic Substances, Environmental Protection Agency, 401 M St., SW., Washington, DC 20460, and from the American Society for Testing and Materials (ASTM), 1916 Race Street, Philadelphia PA 19103.

(2) *Preparation of reagents and solutions*—(i) *Solvents.* For column elution and preparation of buffers, mix chromatographic or reagent grade methanol and water in an 85:15 v/v ratio.

(ii) *Calibration mixture.* Prepare a standard calibration solution containing 200 mg/L of each of the substances listed in the following Table 1 dissolved in acetone and cyclohexane (3:1 v/v) other suitable solvent. Twenty microliters of this solution injected into the chromatograph should give an adequate recorder response (25 percent of scale) for calibration purposes. However, both the concentration and amount injected may be increased or decreased without affecting the retention times, since $t_R$ is independent of concentration in dilute solutions.

TABLE 1—MEASURED OCTANOL/WATER PARTITION COEFFICIENTS AND TYPICAL HPLC RETENTION TIMES FOR THE CHEMICALS USED IN THE CALIBRATION MIXTURE

| Chemical | Literature log $K_{ow}$ | Measured typical retention time | |
|---|---|---|---|
| | | Minutes | Log minutes |
| Benzene | 2.13 | 4.12 | 0.61 |
| Bromobenzene | 2.99 | 7.09 | 0.85 |
| Biphenyl | 3.76 | 8.85 | 0.95 |
| Bibenzyl | 4.81 | 15.87 | 1.20 |
| p,p'-DDE | 5.69 | 21.96 | 1.34 |
| 2,4,5,2',5'-PCB | 6.11 | 31.58 | 1.50 |

(iii) *Test solution.* Solutions of the test substance(s) are prepared similar to the calibration mixture: by dissolving the substance(s) to be tested in a 3:1 mixture of acetone and cyclohexane. The concentration of the substance(s), as determined by trial and error, should be sufficient to produce a chromatographic peak of at least 25 percent of the recorder scale.

(3) *Performance of the test.* (i) After conditioning the column with 85:15 methanol-water or buffered methanol-water, chromatograph the calibration mixture by injecting 20 microliters of the mixture into the column. Elute the column using a solvent flow rate of about 2.0 ml/min at a pressure of approximately 1200 psi. Determine the retention time for each substance in the mixture. The calibration mixture must be chromatographed daily because the retention time is sensitive to

variations in the flow rate, temperature, solvent ratio, and the retention properties of the column.

(ii) Chromatograph 20 microliters of the test solution(s) directly following column calibration, using an identical flow rate and pump pressure. Determine the retention time(s) for the test substance(s).

(c) *Data and reporting*—(1) *Test report.* (i) Using the measured retention times of the substances in the calibration mixture and the log $K_{ow}$ for each substance in Table 1 under paragraph (b)(2)(ii) of this section make a plot of log $t_R$ *vs.* log $K_{ow}$. From the data used in making this plot compute a linear regression equation of the form:

$$\log K_{ow} = m \log t_R + b$$

i.e., $y = mx + b$. For each set of test conditions (flow rate, pressure) report this equation, its correlation coefficient and the data used in its calculation.

(ii) Calculate an estimated log $K_{ow}$ for each test substance from its retention time and corresponding regression equation. Report the retention time, its logarithm and log $K_{ow}$ along with the above data.

(2) *Specific analytical procedures.* (i) Provide a detailed description of, or reference for, the liquid chromatograph, separation column, and detector.

(ii) Report the temperature at which the test(s) were conducted.

(iii) Give a description of any problems (and their rectification) or changes in the test procedures.

(d) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Hansch, C., Fujita, T. "A Method for the Correlation of Biological Activity and Chemical Structure," *American Chemical Society Journal,* 86:1616 (1964).

(2) Hansch, C., Leo, A. *Substituent Constants for Correlation Analysis in Chemistry and Biology.* (J. Wiley & Sons, New York, 1979).

(3) Lyman, W.J., Reehl, W.F., Rosenblatt, D.H. *Handbook of Chemical Property Estimation Methods: Environmental Behavior of Organic Compounds.* (McGraw-Hill Book Co., New York, 1982).

(4) Neely, W.B., Branson, D.R., Blau, G.E. "Partition Coefficient to Measure Bioconcentration Potential of Organic Chemicals in Fish," *Environmental Science Technology,* 8:113 (1974).

(5) Veith, G.D., Austin, N.M., Morris, R.T. "A Rapid Method for Estimating Log P for Organic Chemicals," *Water Research,* 13:43 (1979).

[50 FR 39252, Sept. 27, 1985, as amended at 53 FR 10391, Mar. 31, 1988; 53 FR 12525, Apr. 15, 1988]

## § 796.1720 Octanol/water partition coefficient, generator column method.

(a) *Introduction*—(1) *Background and purpose.* (i) Since the pioneering work of Fujita and Hansch (1964) under paragraph (d)(2) of this section in the measurement and estimation of the octanol/water partition coefficient ($K_{ow}$), this property has become the cornerstone of a myriad of structure-activity relationships (SAR). Hansch and Leo (1979) under paragraph (d)(3) of this section have used the coefficient extensively for correlating structural changes in drugs with changes observed in biological, biochemical or toxic effects. These correlations are then used to predict the effect of a new drug for which a $K_{ow}$ could be measured.

(ii) In the study of the environmental fate of organic chemicals, the coefficient has become a key parameter. It has been shown to be correlated to water solubility, soil/sediment sorption coefficient, and bioconcentration. The importance of this property to SAR is indicated by its discussion in the first chapter of Lyman, Reehl and Rosenblatt's (1982) under paragraph (d)(4) of this section comprehensive compendium of methods for estimating the behavior of organic compounds in the environment. These authors consider the measurement or estimation of the coefficient to be the necessary first step in assessing the fate of new chemicals.

(iii) Of the three properties that can be estimated from $K_{ow}$, water solubility is the most important because it affects both the fate and transport of chemicals. For example, highly soluble chemicals become quickly distributed by the hydrologic cycle, have low sorption coefficients for soils and sediments, and tend to be more easily degraded by microorganisms. In addition, chemical transformation processes such as hydrolysis, direct photolysis, indirect photolysis (oxidation) and tend to occur more readily if a compound is soluble.

(iv) Direct correlations between $K_{ow}$ and both the soil/sediment sorption coefficient and the bioconcentration factor are to be expected. In these cases compounds that are more soluble in octanol (more hydrophobic) would be expected to partition out of the water and into the organic portion of soils/sediments and into lipophilic tissue. The relationship between $K_{ow}$ and the bioconcentration factor, as developed by Neely et al. (1974) under paragraph (d)(7) of this section, and other similar relationships, are the principal means of estimating bioconcentration factors. These factors are then used to predict the potential for a chemical to accumulate in living tissue.

(v) This test method describes a method for determining the octanol/water partition coefficient based on the dynamic coupled column liquid chromatographic technique, a technique commonly referred to as the generator column method. This method was the basis for a previous test method for water solubility, § 796.1860 and closely follows that section. The method described herein can be used in place of the standard shake-flask method described in § 796.1550 for compounds with a log $K_{ow}$ greater than 1.0.

(2) *Definitions and units.* (i) The octanol/water partition coefficient ($K_{ow}$) is defined as the ratio of the molar concentrations of a chemical in n-octanol and water, in dilute solution. The coefficient $K_{ow}$ is a constant for a given chemical at a given temperature. Since $K_{ow}$ is the ratio of two molar concentrations, it is a dimensionless quantity. Sometimes $K_{ow}$ is reported as log $K_{ow}$. The mathematical statement of $K_{ow}$ is:

*Equation 1*

$$K_{ow} = \frac{C_{octanol}}{C_{water}},$$

where $C_{octanol}$ and $C_{water}$ are the molar concentration of the solute in n-octanol and water, respectively, at a given temperature. This test procedure determines $K_{ow}$ at 25±0.05 °C.

(ii) A "generator column" is used to partition the test substance between the octanol and water phases. The column in Figure 1 under paragraph (b)(1)(i)(A)(*2*) of this section is packed with a solid support and is coated with the test substance at a fixed concentration in n-octanol. The test substance is eluted from the column with water and the aqueous solution leaving the column represents the equilibrium concentration of the test substance that has partitioned from the octanol phase into the water phase. Preparation of the generator column is described under paragraph (b)(1)(i) of this section.

(iii) An "extractor column" is used to extract the solute from the aqueous solution produced by the generator column. After extraction onto a bonded chromatographic support, the solute is eluted with a solvent/water mixture and subsequently analyzed by high pressure liquid chromatography (HPLC). A detailed description of the preparation of the extractor column is given in paragraph (b)(1)(i) of this section.

(iv) The "sample loop" is a 1/16 in. O.D. (1.6mm) stainless steel tube with an internal volume between 20 and 50 µL. The loop is attached to the sample injection valve of the HPLC and is used to inject standard solutions into the mobile phase of the HPLC when determining the response factor for the recording integrator. The exact volume of the loop must be determined as described in paragraph (b)(3)(ii)(C)(*1*) of this section when the HPLC method is used.

(v) The "response factor" (RF) is the solute concentration required to give a one unit area chromatographic peak or one unit output from the HPLC recording integrator at a particular recorder and detector attenuation. The factor is required to convert from units of area to units of concentration. The determination of the response factor is given in paragraph (b)(3)(ii)(C)(*2*) of this section.

(3) *Principle of the test method.* (i) This test method is based on the dynamic coupled column liquid chromatographic (DCCLC) technique for determining the aqueous solubility of organic compounds that was initially developed by May et al. (1978) under paragraphs (d) (5) and (6) of this section, modified by DeVoe et al. (1981) under paragraph (d)(1) of this section, and finalized by Wasik et al. (1981) under paragraph (d)(8) of this section. The DCCLC technique utilizes a generator column, extractor column and HPLC coupled or interconnected to provide a continuous closed flow system. Aqueous solutions of the test compound are produced by pumping water through the generator column that is packed with a solid support coated with an approximately 1.0 percent (w/w) solution of the compound in octanol. The aqueous solution leaving the column represents the equilibrium concentration of the test chemical which has partitioned from the octanol phase into the water phase. The compound is extracted from the aqueous solution onto an extractor column, then eluted from the extractor column with a solvent/water mixture and subsequently analyzed by HPLC using an ultraviolet absorption detector operating at a suitable wavelength. Chromatogram peaks are recorded and integrated using a recording integrator. The concentration of the compound in the effluent from the generator column is determined from the mass of the compound (solute) extracted from a measured volume of water (solvent). The octanol/water partition coefficient is calculated from the ratio of the molar concentration of the solute in the 1.0 percent (w/w) octanol and molar concentration of the solute in water as determined using the generator column technique.

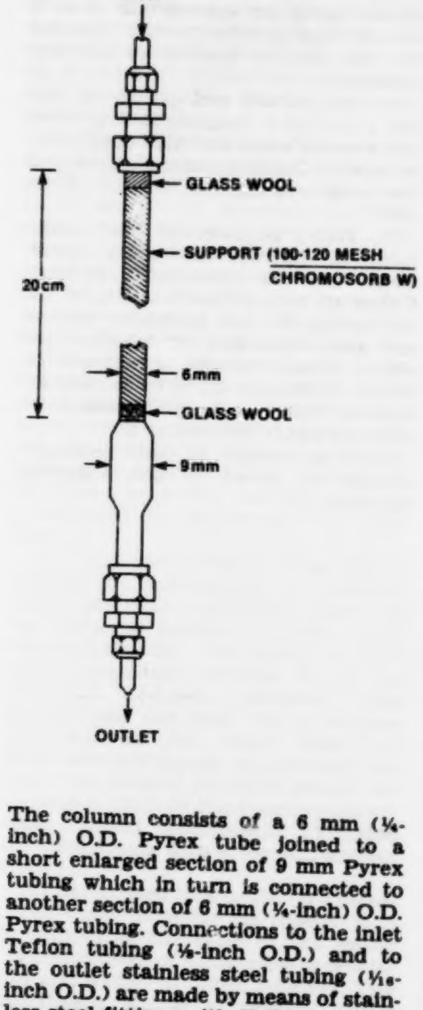
(ii) Since the HPLC method is only applicable to compounds that absorb in the ultraviolet, an alternate gas chromatographic (GC) method is used for those compounds that do not absorb in the ultraviolet. In the GC method the saturated solutions produced in the generator column are extracted using an appropriate organic solvent that is subsequently injected into the GC for analysis of the test compound.

(4) *Applicability and specificity.* The test guideline is designed to determine the octanol/water partition coefficient of solid or liquid organic chemicals in the range log $K_{ow}$ 1.0 to >6.0 (10 to $>10^6$).

(b) *Test procedure*—(1) *Test conditions*—(i) *Special laboratory equipment.* (A) (*1*) Generator column—Either of two different methods for connecting to the generator column are used depending on whether the eluted aqueous phase is analyzed by HPLC (Procedure A) or by solvent extraction followed by GC analysis of solvent extract (Procedure B).

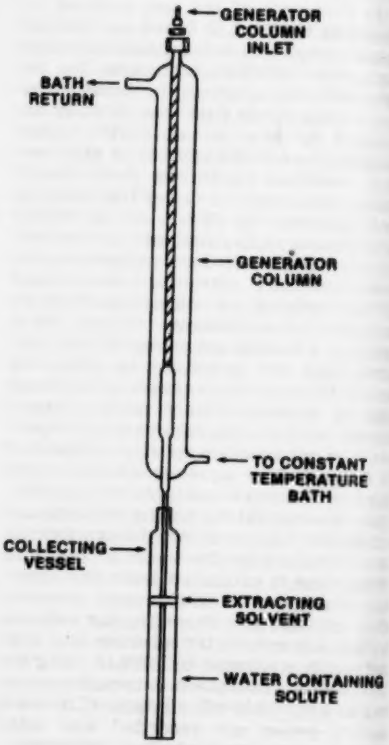
(*2*) The design of the generator column is shown in the following Figure 1:

Figure 1—Generator Column


INLET
GLASS WOOL
SUPPORT (100-120 MESH CHROMOSORB W)
20cm
6mm
GLASS WOOL
9mm
OUTLET

The column consists of a 6 mm (¼-inch) O.D. Pyrex tube joined to a short enlarged section of 9 mm Pyrex tubing which in turn is connected to another section of 6 mm (¼-inch) O.D. Pyrex tubing. Connections to the inlet Teflon tubing (⅛-inch O.D.) and to the outlet stainless steel tubing (1⁄16-inch O.D.) are made by means of stainless steel fittings with Teflon ferrules. The column is enclosed in a water jacket for temperature control as shown in the following Figure 2:

Figure 2—Setup Showing Generator Column Enclosed in a Water Jacket and Overall Arrangement of the Apparatus Used in GC Method


GENERATOR COLUMN INLET
BATH RETURN
GENERATOR COLUMN
TO CONSTANT TEMPERATURE BATH
COLLECTING VESSEL
EXTRACTING SOLVENT
WATER CONTAINING SOLUTE

(B) Constant temperature bath with circulation pump-bath and capable of controlling temperature to 25±0.05 °C. (Procedure A and B);

(C) High pressure liquid chromatograph equipped with an ultraviolet absorption detector operating at a suitable wavelength and a recording integrator (Procedure A);

(D) Extractor column—6.6 x 0.6 cm stainless steel tube with end fittings containing 5 micron frits filled with a superficially porous phase packing

(Bondapack $C_{18}$ Corasil: Water Associates) (Procedure A);

(E) Two 6-port high pressure rotary switching valves (Procedure A);

(F) Collection vessel under paragraph (b)(1)(i)(A)(*2*) Figure 2 of this section 8 x ¾ inch section of Pyrex tubing with a flat bottom connected to a short section of ⅜-inch O.D. borosilicate glass tubing. The collecting vessel is sealed with a ⅜-inch Teflon cap fitting (Procedure B); and

(G) Gas chromatograph equipped with a detector sensitive to the solute of interest (Procedure B).

(ii) *Purity of octanol and water.* Purified n-octanol, described in paragraph (b)(2) of this section, and water meeting ASTM Type II standards, or an equivalent grade, are recommended to minimize the effects of dissolved salts and other impurities. ASTM Type II water is described in ASTM D 1193-77 (Reapproved 1983), "Standard Specification for Reagent Water". ASTM D 1193-77 (Reapproved 1983) is available for inspection at the Office of the Federal Register, Rm. 8401, 1100 L St., NW., Washington, DC. This incorporation by reference was approved by the Director of the Office of the Federal Register. This material is incorporated as it exists on the date of approval and a notice of any change in this material will be published in the FEDERAL REGISTER. Copies of the incorporated material may be obtained from the TSCA Public Docket Office (TS-793), Rm. NE-G004, Office of Toxic Substances, Environmental Protection Agency, 401 M St., SW., Washington, DC 20460, or from the American Society for Testing and Materials (ASTM), 1916 Race Street, Philadelphia, PA 19103.

(iii) *Purity of solvents.* It is important that all solvents used in this method be reagent or HPLC grade and contain no impurities which could interfere with the determination of the test compound.

(iv) *Reference compounds.* In order to ensure that the HPLC system is working properly, at least two of the following five reference compounds should be run. The values obtained should be within ±0.1 log unit of the reference value

| | Log $K_{ow}$ |
|---|---|
| n-Propylacetate | 1.24 |
| 2-Heptanone | 1.98 |
| Naphthalene | 3.34 |
| *n*-Propylbenzene | 3.69 |
| *n*-Octane | 5.18 |

Reference compounds should be reagent or HPLC grade to avoid interferences by impurities.

(2) *Preparation of reagents and solutions*—(i) *Octanol and Water.* Very pure *n*-octanol can be obtained as follows: wash pure *n*-octanol (minimum 98 percent pure) sequentially with 0.1N $H_2SO_4$, with 0.1N NaOH, then with distilled water until neutral. Dry the *n*-octanol with magnesium sulfate and distill twice in a good distillation column under reduced pressure [b.p. about 80 °C at 0.27 kPa (2 torr)]. The octanol produced should be at least 99.9 percent pure. Alternatively, a grade equivalent to Fisher Scientific Co. No. A-402 "Certified Octanol-1" can be used. Distilled or reagent grade water should be used throughout the test procedure; ASTM Type II water as described in ASTM D 1193-77 (Reapproved 1983), "Standard Specification for Water", is recommended. ASTM D1193-77 (Reapproved 1983) is available for inspection at the Office of the Federal Register, Rm. 8401, 1100 L St., NW., Washington, DC. This incorporation by reference was approved by the Director of the Office of the Federal Register. This material is incorporated as it exists on the date of approval and a notice of any change in this material will be published in the FEDERAL REGISTER. Copies of the incorporated material may be obtained from the TSCA Public Docket Office (TS-793), Rm. NE-G004, Office of Toxic Substances, Environmental Protection Agency, 401 M St., SW., Washington, DC 20460, or from the American Society for Testing and Materials (ASTM), 1916 Race Street, Philadelphia, Pa 19103.

(ii) [Reserved]

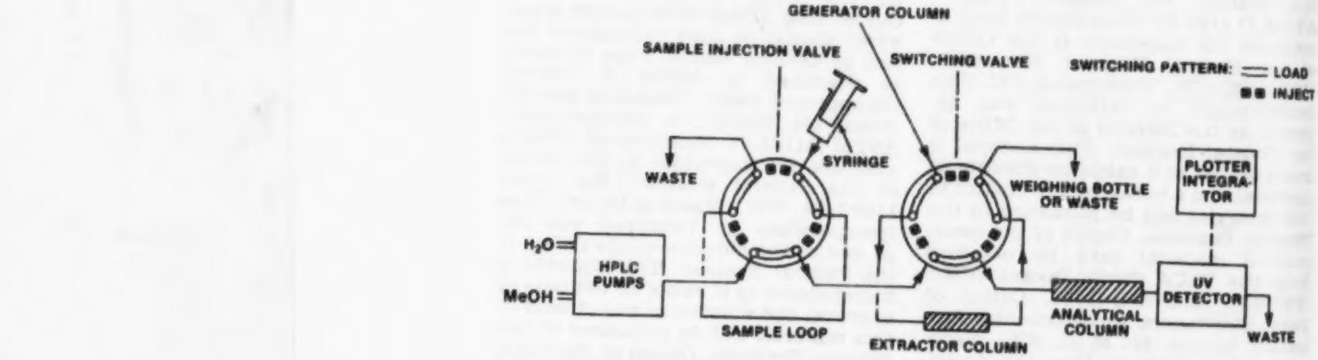
(3) *Performance of the Test.* Initially, an approximately 1.0 percent (w/w) solution of the test substance in *n*-octanol is prepared. Precise measurement of the solute concentration in this solution is required for the $K_{ow}$

calculation. Subsequently, the 1.0 percent (w/w) solution is coated on the generator column and using either Procedure A or B as described below, the molar concentration of the test substance in reagent grade water is determined.

(i) *Test solution.* The test solution consists of an approximately 1.0 percent (w/w) solution of the test substance in octanol. A sufficient quantity (about 10-20 ml) of the test solution should be prepared to coat the generator column. The solution is prepared by accurately weighing out, using a tared bottle, quantities of both the test substance and octanol required to make a 1.0 percent (w/w) solution. When the weights are measured precisely (to the nearest 0.1 mg, knowing the density of octanol (0.827 g/ml at 25 °C), then the molar concentration of the test substance in the octanol is sufficiently accurate for the purposes of the test procedure. If desired, however, a separate analytical determination (e.g., by GC) may be used to check the concentration in the test solution. If storage is required, the test solution should be kept stoppered to prevent volatilization of the test chemical.

(ii) *Procedure A—HPLC Method.* (A) Procedure A covers the determination of the aqueous solubility of compounds which absorb in the ultraviolet. The HPLC analytical system is shown schematically in the following Figure 3:

FIGURE 3—SCHEMATIC OF HPLC—GENERATOR COLUMN FLOW SYSTEM



Two reciprocating piston pumps deliver the mobile phase (water or solvent/water mixture) through two 6-port high pressure rotary valves and a 30 x 0.6 cm $C_{18}$ analytical column to an ultraviolet absorption detector operating at a suitable wavelength. Chromatogram peaks are recorded and integrated with a recording integrator. One of the 6-port valves is the sample injection valve used for injecting samples of standard solutions of the solute in an appropriate concentration for determining response factors or standard solutions of basic chromate for determining the sample loop volume. The other 6-port valve in the system serves as a switching valve for the extractor column which is used to remove solute from the aqueous solutions.

(B) The general procedure for analyzing the aqueous phase after equilibration is as follows; a detailed procedure is given in part d. of this section:

(*1*) Direct the aqueous solution from the generator column to "Waste" in

Figure 3 under paragraph (b)(3)(ii)(A) of this section with the switching valve in the inject position in order to equilibrate internal surfaces with the solution, thus insuring that the analyzed sample would not be depleted by solute adsorption on surfaces upstream from the valve.

(*2*) At the same time, water is pumped from the HPLC pumps in order to displace the solvent from the extractor column.

(*3*) The switching valve is next changed to the load position to divert a sample of the solution from the generator column through the extractor column, and the liquid leaving the extractor column is collected in a tared weighing bottle. During this extraction step, the HPLC mobile phase is changed to a solvent/water mixture to condition the analytical column.

(*4*) After the desired volume of sample is extracted, the switching valve is returned to the inject position for elution from the extractor column and analysis. Provided that all of the solute was adsorbed by the extractor column during the extraction step, the chromatographic peak represents all of the solute in the extracted sample.

(*5*) The solute concentration in the aqueous phase is calculated from the peak area, the weight of the extracted liquid collected in the weighing bottle, and the response factor.

(C)(*1*) *Determination of the Sample Loop Volume.* Accurate measurement of the sample loop may be accomplished by using the spectrophotometric method of Devoe et al. (1981) under paragraph (d)(1) of this section. For this method measure absorbance, $A_{loop}$, at 373 nm for at least three solutions, each of which is prepared by collecting from the sample valve an appropriate number, n, of loopfuls of an aqueous stock solution of $K_2CrO_4$ (1.3 percent by weight) and diluting to 50 mL with 0.2 percent KOH. (For a 20 μL loop, use n=5; for a 50 μL loop, use n=2.) Also measure the absorbance, $A_{stock}$, of the same stock solution after diluting 1:500 with 0.2 percent KOH. Calculate the loop volume to the nearest 0.1 μL using the relation:

$$V_{loop}=(A_{loop}/A_{stock})(10^{-4}/n)$$

(*2*) *Determination of the response factor (RF).* (*i*) For all determinations adjust the mobile phase solvent/water ratio and flow rate to obtain a reasonable retention time on the HPLC column. For example, typical concentrations of organic solvent in the mobile phase range from 50 to 100 percent while flow rates range from 1 to 3 mL/min; these conditions often give a 3 to 5 minute retention time.

(*ii*) Prepare standard solutions of known concentrations of the solute in a suitable solvent. Concentrations must give a recorder response within the maximum response of the detector. Inject samples of each standard solution into the HPLC system using the calibrated sample loop. Obtain an average peak area from at least three injections of each standard sample at a set detector absorbance unit full scale (AUFS), i.e., at the same absorbance scale attenuation setting.

(*iii*) Calculate the response factor from the following equation:

$$\text{Response Factor (RF)}=\frac{\text{Concentration (M)}}{\text{(Average Area) (AUFS)}}.$$

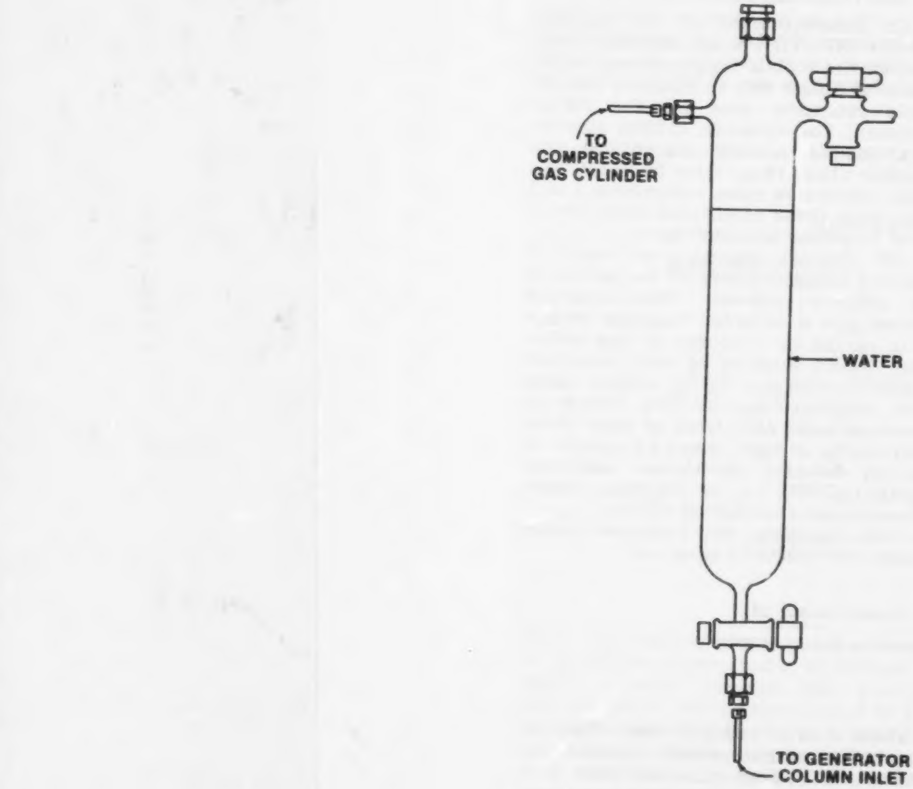
(*3*) *Loading of the generator column.* (*i*) The design of the generator column was described in paragraph (b)(1)(i) of this section and is shown in Figure 1 under paragraph (b)(1)(i)(A)(*2*) of this section. To pack the column, a plug of silanized glass wool is inserted into one end of the 6 mm Pyrex tubing. Silanized diatomaceous silica support (about 0.5g of 100–120 mesh Chromosorb W chromatographic support material) is poured into the tube with tapping and retained with a second plug of silanized glass wool.

(*ii*) The column is loaded by pulling the test solution through the dry support with gentle suction and then allowing the excess solution to drain out. After loading the column, draw water up through the column to remove any entrapped air.

(*4*) *Analysis of the solute.* Use the following procedure to collect and analyze the solute:

(*i*) Pump water to the generator column by means of a minipump or pressurized water reservoir as shown in the following Figure 4:

FIGURE 4—WATER RESERVOIR FOR GC METHOD



With the switching valve in Figure 3 under paragraph (b)(3)(ii)(A) of this section in the inject position (i.e., water to waste), pump water through the generator column at a flow rate of approximately 1 mL/min for approximately 15 minutes to bring the system into equilibrium.

(*ii*) Flush out the organic solvent that remains in the system from previous runs by changing the mobile phase to 100 percent $H_2O$ and allowing the water to reach the HPLC detector as indicated by a negative reading. As soon as this occurs, place a 25 mL weighing bottle (weighed to the nearest mg) at the waste position and immediately turn the switching valve to the load position.

(*iii*) Collect an amount of water from the generator column (as determined by trial and error) in the weighing bottle, corresponding to the amount of solute adsorbed by the extractor column that gives a reasonable detector response. During this extraction step, switch back to the original HPLC mobile phase composition, i.e., solvent/water mixture, to condition the HPLC analytical column.

(*iv*) After the desired volume of sample has been extracted, turn the switching valve back to the inject position in Figure 3 under paragraph (b)(3)(ii)(A) of this section. As soon as switching valve is turned to the inject position, remove the weighing bottle, cap it and replace it with the waste container; at the same time turn on the recording integrator. The solvent/water mobile phase will elute the solute from the extractor column and transfer the solute to the HPLC analytical column.

(*v*) Determine the weight of water collected to the nearest mg and record the corresponding peak area. Using the same AUFS setting repeat the analysis of the solute at least two more times and determine the average ratio of peak area to grams of water collected. Calculate the solute solubility in water using the following equation:

$$S=(997\ g/L)(RF)(V_{loop})(AUFS)(R)$$

where

$S$=solubility (M)
$RF$=response factor
$V_{loop}$=sample loop volume (L)
$R$=ratio of area to grams of water.

(iii) *Procedure B—GC Method.* In the GC method, aqueous solutions from the generator column enter a collecting vessel in Figure 2 under paragraph (b)(1)(i)(A)(*2*) of this section containing a known weight of extracting solvent which is immiscible in water. The outlet of the generator column is positioned such that the aqueous phase always enters below the extracting solvent. After the aqueous phase is collected, the collecting vessel is stoppered and the quantity of aqueous phase is determined by weighing. The solvent and the aqueous phase are equilibrated by slowly rotating the collecting vessel. A small amount of the extracting solvent is then removed and injected into a gas chromatograph equipped with an appropriate detector. The solute concentration in the aqueous phase is determined from a calibration curve constructed using known concentrations of the solute.

(A) *Determination of calibration curve.* (*1*) Prepare solute standard solutions of concentrations covering the expected range of the solute solubility. Select a column and optimum GC operating conditions for resolution between the solute and solvent and the solute and extracting solvent. Inject a known volume of each standard solution into the injection port of the GC. For each standard solution determine the average of the ratio R of peak area to volume (in $\mu$L) for the chromatographic peak of interest from at least three separate injections.

(*2*) After running all the standard solutions, determine the coefficients, a and b, using linear regression analysis on the equation of concentration (C) vs. R in the form

$$C = aR + b.$$

(B) *Loading of the generator column.* The generator column is packed and loaded with solute in the same manner as for the HPLC method in paragraph (b)(3)(ii) of this section. As shown in Figure 2 under paragraph (b)(1)(i)(A)(*2*) of this section, attach approximately 20 cm of straight stainless steel tubing to the bottom of the generator column. Connect the top of the generator column to a water reservoir in Figure 4 under paragraph (b)(3)(ii)(C)(*4*)(*i*) of this section using Teflon tubing. Use air or nitrogen pressure (5 PSI) from an air or nitrogen cylinder to force water from the reservoir through the column. Collect water in an Erlenmeyer flask for approximately 15 minutes while the solute concentration in water equilibrates; longer time may be required for less soluble compounds.

(C) *Collection and extraction of the solute.* During the equilibration time, add a known weight of extracting solvent to a collection vessel which can be capped. The extracting solvent should cover the bottom of the collection vessel to a depth sufficient to submerge the collecting tube but still maintain 100:1 water/solvent ratio. Record the weight (to the nearest mg) of a collection vessel with cap and extracting solvent. Place the collection vessel under the generator column so that water from the collecting tube enters below the level of the extracting solvent in Figure 2 under paragraph (b)(1)(i)(A)(*2*) of this section. When the collection vessel is filled, remove it from under the generator column, replace cap, and weigh the filled vessel. Determine the weight of water collected. Before analyzing for the solute, gently rotate the collection vessel contents for approximately 30 min., controlling the rate of rotation so as not to form an emulsion; rotating the flask end over end five times per minute is sufficient.

(D) Analysis of the solute. (*1*) After rotating, allow the collection vessel to stand for approximately 30 minutes; then remove a known volume of the extracting solvent from the vessel using a microliter syringe and inject in into the G.C. Record the ratio of peak area to volume injected and, from the regression equation of the calibration line, determine the concentration of solute in the extracting solvent. The molar concentration of solute in water C(M) is determined from the following equation

$$C(M) = (C_{es}) \frac{d_{H_2O}}{d_{es}} \frac{g_{es}}{g_{H_2O}}$$

where $C_{es}$ is the molar concentration of solute in extracting solvent, $d_{H_2O}$ and $d_{es}$ are the densities in gm/ml of water and extracting solvent, respectively, and $g_{es}$ and $g_{H_2O}$ are the grams of extracting solvent and water, respectively, contained in the collection vessels.

(*2*) Make replicate injections from each collecting vessel to determine the average solute concentration in water for each vessel. To make sure the generator column has reached equilibrium, run at least two additional (for a total of three) collection vessels and analyze the extracted solute as described above. Calculate (CM) from the average solute concentration in the three vessels.

(iv) *Analysis of reference compounds.* Prior to analyzing the test solution, make duplicate runs on at least two of the five reference compounds given in paragraph (b)(1)(iv) of this section. When using the reference compounds, follow the same procedure previously described for preparing the test solution and running the test. If the average value obtained for each compound is 0.1 log unit of the reference value, then the test procedure and HPLC system are functioning properly; if not a thorough checking over of the HPLC and careful adherence to the test procedures should be done to correct the discrepancy.

(v) *Modification of procedures for potential problems—Decomposition of the test compound.* If the test compound decomposes in one or more of the aqueous solvents required during the period of the test at a rate such that an accurate value for water solubility cannot be obtained, then it will be necessary to carry out detailed transformation studies; e.g., hydrolysis under § 796.3500. If decomposition is due to aqueous photolysis, then it will be necessary to carry out water solubility studies in the dark, under red or yellow lights, or by any other suitable method to eliminate this transformation process.

(c) *Data and reporting*—(1) *Test report.* (i) For the test solution, report the weights to the nearest 0.1 mg of the test substance and octanol. Also report the weight percent and molar concentration of the test substance in the octanol, the density of *n*-octanol at 25 °C is 0.827 gm/ml.

(ii) For each run provide the molar concentration of the test substance in water for each of three determinations, the mean value, and the standard deviation.

(iii) For each of the three determinations calculate the octanol/water partition coefficient as the ratio of the molar concentration of the test substance in octanol to the molar concentration in water. Also calculate and report the mean $K_{ow}$ and its standard deviation. Values of $K_{ow}$ may be reported as their logarithms if desired.

(iv) Report the temperature (± 0.05 °C) at which the generator column was controlled during the test.

(v) For each reference compound report the individual values of log $K_{ow}$ and the average of the two runs.

(vi) For compounds that decompose at a rate such that a precise value for the solubility cannot be obtained, provide a statement to that effect.

(2) *Specific analytical, calibration and recovery procedures.* (i)(A) For the HPLC method describe and/or report:

(*1*) The method used to determine the sample loop volume and the average and standard deviation of that volume.

(*2*) The average and standard deviation of the response factor.

(*3*) Any changes made or problems encountered in the test procedures.

(B) For the GC method report:

(*1*) The column and GC operating conditions of temperature and flow rate.

(*2*) The average and standard deviation of the average area µL obtained for each of the standard solutions.

(*3*) The form of the regression equation obtained in the calibration procedure.

(*4*) The extracting solvent used.

(*5*) The average and standard deviation of solute concentration in each collection vessel.

(*6*) Any changes made or problems encountered in the test procedure.

(C) If the concentration of the test substance in *n*-octanol is determined by an independent analytical method such as gas chromatography, provide a complete description of the method.

(ii) [Reserved]

(d) *References.* For additional background information on this test guideline the following reference should be consulted:

(1) DeVoe, H., Miller, M.M., Wasik, S.P. "Generator Columns and High Pressure Liquid Chromatography for Determining Aqueous Solubilities and Octanol-Water Partition Coefficients of Hydrophobic Substances," *Journal of Research of the National Bureau of Standards,* 86:361-366 (1981).

(2) Fujita, T., Iwasa, J., Hansch, C. "A New Substituent Constant, Derived from Partition Coefficients." *American Chemical Society Journal,* 86:5175 (1964).

(3) Hansch, C., Leo, A. *Substituent Constants for Correlation Analysis in Chemistry and Biology.* (New York: J. Wiley & Sons, 1979).

(4) Lyman, W.J., Reehl, W.F., Rosenblatt, D.H. *Handbook of Chemical Property Estimation Methods, Environmental Behavior of Organic Compounds.* (McGraw Hill Book Co., New York, 1982).

(5) May, W.E., Wasik, S.P., Freeman, D.H. "Determination of the Aqueous Solubility of Polynuclear Aromatic Hydrocarbons by a Coupled Column Liquid Chromatographic Technique," *Analytical Chemistry,* 50:175-179 (1978).

(6) May, W.E., Wasik, S.P., Freeman, D.H. "Determination of the Solubility Behavior of Some Polycyclic Aromatic Hydrocarbons in Water," *Analytical Chemistry,* 50:997-1000 (1978).

(7) Neely, W.B., Bronson, D.R., Blau, G.E. "Partition Coefficient to Measure Bioconcentration Potential of Organic Chemicals in Fish," *Environmental Science and Technology,* 8:113 (1974).

(8) Wasik, S.P., Tewari, Y.B., Miller, M.M., Martire, D.E. "Octanol/Water Partition Coefficient and Aqueous Solubilities of Organic Compounds," Report NBSIR 81-2406, National Bureau of Standards, U.S. Department of Commerce, Washington, DC. (1981).

[50 FR 39252, Sept. 27, 1985, as amended at 53 FR 10391, Mar. 31, 1988; 53 FR 12525, Apr. 15, 1988]

### § 796.1840 Water solubility.

(a) *Introduction*—(1) *Background and purpose.* (i) The water solubility of a compound can be defined as the equilibrium concentration of the compound in a saturated aqueous solution at a given temperature and pressure. The water solubility of a chemical is an important factor in determining the environmental movement and distribution of any substance. Chemicals that are relatively water soluble are more likely to be widely distributed by the hydrologic cycle than those which are relatively insoluble.

(ii) Water provides the medium in which many organisms live, and water is a major component of the internal environment of all living organisms (except for dormant stages of certain life forms). Even organisms which are adapted to life in a gaseous environment require water for normal functioning. Water is thus the medium through which most other chemicals are transported to and into living cells. As a result, the extent to which chemicals dissolve in water will be a major determinant for movement through the environment and entry into living systems.

(iii) The water solubility of a chemical has an effect on its adsorption on and desorption from soils and sediments and on volatilization from aqueous media. The more soluble a chemical substance is, the more likely it is to desorb from soils and sediments and the less likely it is to volatilize from water. The extent of chemical transformations via hydrolysis, photolysis, oxidation, reduction, and biodegradation in water depends on the chemical being soluble in water (i.e., homogeneous kinetics). Finally, the design of most chemical tests and many ecological and health tests requires precise knowledge of the water solubility of the chemical to be tested.

(iv) Procedures in this test guideline have been described to enable sponsors to determine the water solubility for solid and liquid organic compounds.

(2) *Definitions and units.* (i) "Colloidal dispersion" is a mixture resembling a true solution but containing one or more substances that are finely divided but large enough to prevent passage through a semipermeable membrane. It consists of particles which are larger than molecules, which settle out very slowly with time,

which scatter a beam of light, and which are too small for resolution with an ordinary light microscope.

(ii) A "concentration vs. time study" results in a graph which plots the measured concentration of a given compound in a solution as a function of elapsed time. Usually, it provides a more reliable determination of equilibrium water solubility of hydrophobic compounds than can be obtained by single measurements of separate samples.

(iii) "Concentration" of a solution is the amount of solute in a given amount of solvent and can be expressed as a weight/weight or weight/volume relationship. The conversion from a weight relationship to one of volume incorporates density as a factor. For dilute aqueous solutions, the density of the solvent is approximately equal to the density of the solutions; thus, concentrations in mg/dm$^3$ are approximately equal to $10^{-3}$ g/$10^3$ g or parts per million (ppm); ones in μg/dm$^3$ are approximately equal to $10^{-6}$ g/$10^3$ g or parts per billion (ppb). In addition, concentration can be expressed in terms of molarity, normality, molality, and mole fraction. For example, to convert from weight/volume to molarity one incorporates molecular mass as a factor.

(iv) "Density" is the mass of a unit volume of a material. It is a function of temperature, hence the temperature at which it is measured should be specified. For a solid, it is the density of the impermeable portion rather than the bulk density. For solids and liquids, suitable units of measurement are g/cm$^3$. The density of a solution is the mass of a unit volume of the solution and suitable units of measurement are g/cm$^3$.

(v) An "oversaturated (supersaturated) solution" is a solution that contains a greater concentration of a solute than is possible at equilibrium under fixed conditions of temperature and pressure.

(vi) A "saturated solution" is a solution in which the dissolved solute is in equilibrium with an excess of undissolved solute; or a solution in equilibrium such that at a fixed temperature and pressure, the concentration of the solution is at its maximum value and will not change even in the presence o an excess of solute.

(vii) A "solution" is a homogeneou mixture of two or more substance constituting a single phase.

(3) *Principle of the test method.* Th test method is based on the conventional method of preparing saturate aqueous solutions. The method involves the coating of the compound t the walls of a vessel, adding water (i.e. very pure water, buffer solution, or artificial seawater), and determining the concentration of the compound in the water as a function of time at a fixed temperature. When the concentratio reaches a plateau, equilibrium has been achieved, and the water is saturated with the compound. Specific procedures have been incorporated in this test guideline to measure the water solubility of very hydrophobic compounds and to alleviate the problems of colloids and emulsions usually formed.

(4) *Applicability and specificity.* (i) Procedures have been described in this test guideline to determine the saturated water solubility for liquid or solid compounds. The water solubility can be determined in very pure water, buffer solution for compounds that reversibly ionize or protonate, or in artificial seawater as a function of temperature (i.e., in the range of temperatures of environmental concern). Water solubility is usually not useful for gases because their solubility in water is measured when the gas above the water is at a partial pressure of one atmosphere which is several orders of magnitude greater than those existing under environmental conditions. A more important parameter for gases is Henry's law constant which is the ratio of the vapor pressure of the compound to solution concentration at low partial pressures.

(ii) This test guideline is designed to determine the saturated water solubility of a solid or liquid test chemical in the range infinit to 10 parts per billion (ppb). For chemicals whose solubility is below 10 ppb, the water solubility should be characterized as "less than 10 ppb" with no further quantification.

(b) *Description of the test procedure*—(1) *Test conditions*—(i) *Special* *laboratory equipment.* (A) A thermostatic bath with temperature control (±1 °C) in the approximate range of 5-30 °C.

(B) An ultracentrifuge with temperature control (±1 °C) in the approximate range of 5-30 °C and capable of obtaining acceleration (G) values to 39,000 or higher.

(C) A pH meter capable of resolving differences of 0.1 pH units or less.

(D) Centrifuge tubes with sealable caps: special glass tubes can be used up to approximately 12,000 G; tubes to be used at G value >12,000 should be made of stainless steel.

(ii) *Purity of water.* Reagent grade water, e.g., water meeting ASTM Type IIA standards or an equivalent grade, is highly recommended to minimize biodegradation and to minimize the effects of dissolved salts on water solubility. ASTM Type IIA water is described in ASTM D 1193-77 (Reapproved 1983), "Standard Specification for Reagent Water". ASTM D 1193-77 (Reapproved 1983) is available for inspection at the Office of the Federal Register, Rm. 8401, 1100 L. St., NW., Washington, DC. This incorporation by reference was approved by the Director of the Office of the Federal Register. This material is incorporated as it exists on the date of approval and a notice of any change in this material will be published in the FEDERAL REGISTER. Copies of the incorporated material may be obtained from the TSCA Public Docket Office (TS-793), Rm. NE-G004, Office of Toxic Substances, Environmental Protection Agency, 401 M St., SW., Washington, DC 20460, or from the American Society for Testing and Materials (ASTM), 1916 Race Street, Philadelphia, Pa 19103.

(iii) *Purity of solvents.* All solvents used for coating test compounds on the walls of vessels and in separation and analytical technique shall be reagent grade and contain no impurities which will interfere with the determination of the test compound.

(iv) *Seawater.* It is recommended that artificial seawater be used to determine the saturated water solubility in seawater. The preparation of artificial seawater is described in paragraph (b)(2)(ii) of this section.

(v) *Agitation and equilibration time.* Contact time of test compounds with water shall be sufficient to obtain a saturated solution. The length of time necessary will depend upon such variables as the size of the vessel, the extent and degree of agitation, the properties of the compound and particle size. To increase the rate of solution of hydrophobic compounds, a mild agitation is recommended. For hydrophobic compounds a minimum time of one day is required.

(vi) *Effects of colloids and emulsions: Centrifugation.* (A) Gentle shaking shall be used to minimize the formation of colloids. The presence of colloids and emulsions will lead to solubility values that are higher than those in a true saturated solution. This is a common problem with hydrophobic solids and liquids but can usually be overcome by centrifugation. Centrifugation shall be conducted in tightly sealed tubes that are almost filled to capacity to avoid partitioning with air.

(B) Centrifugation shall be carried out at two or three different G values (minimum of 12,000 G) for at least 30 minutes at 25 °C until concentration changes are small. For hydrophobic compounds (solubility <10 ppm), the acceleration G values shall differ by 10,000 G and include a determination of 39,000 or higher.

(vii) *Effect of pH on solubility.* All experiments shall be carried out at pH's 5.0, 7.0, and 9.0 for any chemical which reversibly ionizes or protonates (e.g., carboxylic acids, phenols, amines). Buffers described in paragraph (b)(2)(i) of this section can be used.

(viii) *Analysis of saturated solutions.* Any suitable analytical method may be used; where practicable, precision should be within ±5 percent. Preferred analytical methods are those that are specific for the compound to be tested, to the exclusion of other compounds. Chromatographic methods which incorporate separation, and therefore, specification, are recommended.

(ix) *Adsorption to glass or other surfaces.* Hydrophobic compounds have a tendency to adsorb to glass or other surfaces, e.g., stainless steel. Thus, when transferring the solution to any glass vessel or container, it is essential to pre-rinse the surfaces of the vessel or container with the solution. Failure to do so will lead to solubility values that are lower than those of true equilibrium water solubility because the compound will adsorb to the unrinsed surface. However, when hydrophobic compounds are extracted with organic solvent, the extraction vessels shall not be pre-rinsed since this would lead to solubility values that are greater than those of true equilibrium water solubility.

(2) *Preparation of reagents and solutions*—(i) *Buffer solutions.* Prepare buffer solutions using reagent grade water as follows:

pH 5.0—To 250 mL of 0.1M potassium hydrogen phthalate add 113 mL of 0.1M sodium hydroxide; adjust the final volume to 500 mL with reagent water.

pH 7.0—To 250 mL of 0.1M potassium dihydrogen phosphate add 145 mL of 0.1M sodium hydroxide; adjust the final volume to 500 mL with reagent gradewater.

pH 9.0—To 250 mL of 0.075M borax add 69 mL of 0.1M HCL; adjust the final volume to 500 mL with reagent gradewater.

Check the pH of each buffer solution with a pH meter at 25 °C and adjust to pH 5.0, 7.0, or 9.0, if necessary. If the pH of the solution has changed by ±0.2 pH units or more after the addition of the test compound, then a more concentrated buffer is required for that pH determination. The sponsor should then choose a more suitable buffer.

(ii) *Artificial seawater.* Add the reagent-grade chemicals listed in the following Table 1 in the specified amounts and order to 890 mL of reagent-grade water. Each chemical shall be dissolved before another one is added.

TABLE 1—CONSTITUENTS OF ARTIFICIAL SEAWATER [1]

| Chemical | Amount |
|---|---|
| NaF | 3 mg |
| $SrCl_2 \cdot 6H_20$ | 20 mg |
| $H_3BO_3$ | 30 mg |
| KBr | 1,000 mg |
| KCl | 700 mg |
| $CaCl_2 \cdot 2H_2O$ | 1.47 g |
| $Na_2SO_4$ | 4.00 g |
| $MgCl_2 \cdot 6H_2O$ | 10.78 g |

TABLE 1—CONSTITUENTS OF ARTIFICIAL SEAWATER [1]—Continued

| Chemical | Amount |
|---|---|
| NaCl | 23.50 g |
| $Na_2SiO_3 \cdot 9H_2O$ | 20 mg |
| $NaHCO_3$ | 200 mg |

[1] If the resulting solution is diluted to 1 cubic decimeter (1 liter), the salinity should be 34 ±0.5 g/kg and the pH 8.0 ±0.2. The desired test salinity is attained by dilution at time of use.

(3) *Performance of the test.* Determine the saturated water solubility of the test compound at 25 °C in reagent grade water or buffer solution, if appropriate. Under certain circumstances, it may be necessary to determine the water solubility of a test compound at 25 °C in artificial seawater. The water solubility can also be determined at other temperatures of environmental concern by adjusting the temperature of the water bath to the appropriate temperature.

(i) *Procedure for the determination of solids and liquids in water at 25 °C.* (A) Dissolve a sufficient amount of the solid compound in suitable volatile organic solvent and coat on the walls of a vessel. Viscous liquids may be coated on vessels in a similar fashion: Nonviscous liquids do not require solvents. Remove the solvent under reduced pressure or with a pure nitrogen gas stream. When all the solvent is removed, add reagent-grade water or, for compounds which reversibly ionize or protonate, the appropriate buffer solution and slowly stir or agitate the mixture under temperature control. Mixing may be accomplished by use of a Teflon-coated stirring bar and shall be continued for a minimum of 24 hours before aliquots are withdrawn. Prior to taking aliquots, the mixture shall be left to stand at constant temperature for at least 1 hour to permit separation of any small particles. To determine the concentration of the compound in the aqueous phase, aliquots shall be centrifuged at two or three different G values (minimum of 12,000 G) for at least 30 minutes at 25 °C until concentration changes are small. The concentration value so obtained is plotted against the time of mixing. At a later time, aliquots are again taken and analyzed in the same fashion to produce another data point on a concentration versus time plot. When the concentration reaches a plateau, equilibrium is assumed. For hydrophobic compounds (solubility $<10$ ppm) it is extremely important that the acceleration (G) values differ by 10,000 G and include a determination at 39,000 G or higher.

(B) For more soluble compounds (solubility $>$ 500 ppm) coating the walls of the vessel is not necessary and filtration may be substituted for centrifugation. Use filters which are adequate to remove suspended particles. If the concentration of the solute exceeds 10 g/dm$^3$, then determine the density of the solution. This can be done by weighing known volumes of the solution at the same temperature as the constant temperature bath. Sufficient solution shall be used so that each determination is made on a fresh aliquot. Carry out solubility and density experiments in triplicate.

(ii) *Modification of Procedures for Potential Problems*—(A) *Interference of Soluble Impurities.* Interference by soluble impurities in the test sample can be avoided by the use of an analytical technique that is specific to the compound being tested. If this is not practical, interferences can sometimes be minimized by repeatedly preparing saturated solutions from the same sample chemical until the concentration of the impurity has been depleted.

(B) *Decomposition of the test compound.* If the test compound decomposes in one or more of the aqueous solvents required during the period of the test at a rate such that an accurate value for water solubility cannot be obtained, then it shall be necessary to carry out detailed transformation studies e.g., hydrolysis. If decomposition is due to aqueous photolysis, then it shall be necessary to carry out the water solubility studies in the dark, under red or yellow lights, or by any other suitable method to eliminate this transformation process.

(c) *Data and reporting*—(1) *Test report.* (i) For each set of conditions, (e.g., temperature, pure water, buffer solution, artificial seawater) required for the study, provide the water solubility value for each of three determinations, the mean value, and the standard deviation.

(ii) For compounds that decompose at a rate such that a precise value for the water solubility cannot be obtained, provide a statement to that effect.

(iii) For compounds with water solubility below 10 ppb, report the value as "less than 10 ppb."

(iv) For compounds with water solubility greater than 10 g/dm$^3$, report the density of the solution at each required temperature.

(2) *Specific analytical and recovery procedures.* (i) Provide a detailed description or references for the analytical procedure used, including the calibration data and precision; and

(ii) If extraction methods were used to separate the solute from the aqueous solution provide a description of the extraction method as well as the recovery data.

[50 FR 39252, Sept. 27, 1985, as amended at 52 FR 19057, May 20, 1987; 53 FR 10391, Mar. 31, 1988; 53 FR 12525, Apr. 15, 1988]

**§ 796.1860 Water solubility (generator column method).**

(a) *Introduction*—(1) *Background and purpose.* (i) The water solubility of a chemical is defined as the equilibrium concentration of the chemical in a saturated aqueous solution at a given temperature and pressure. The aqueous phase solubility is an important factor in governing the movement, distribution, and rate of degradation of chemicals in the environment. Substances that are relatively water soluble are more likely to be widely distributed by the hydrologic cycle than those which are relatively insoluble. Furthermore, substances with higher water solubility are more likely to undergo microbial or chemical degradation in the environment because dissolution makes them "available" to interact and, therefore, react with other chemicals and microorganisms. Both the extent and rate of degradation via hydrolysis, photolysis, oxidation, reduction, and biodegradation depend on a chemical being soluble in water (i.e., homogeneous kinetics).

(ii) Water provides the medium in which many organisms live, and water is a major component of the internal environment of all living organisms (except for dormant stages of certain life forms). Even organisms which are adapted to life in a gaseous environment require water for normal functioning. Water is thus the medium through which most other chemicals are transported to and into living cells. As a result, the extent to which chemicals dissolve in water will be a major determinant for movement through the environment and entry into living systems.

(iii) The water solubility of a chemical also has an effect on its adsorption on and desorption from soils and sediments, and on volatilization from aqueous media. The more soluble a chemical substance is, the less likely it is to adsorb to soils and sediments and the less likely it is to volatilize from water. Finally, the design of most chemical tests and many ecological and health tests requires precise knowledge of the water solubility of the chemical to be tested.

(2) *Definitions and units.* (i) "Concentration" of a solution is the amount of solute in a given amount of solvent or solution and can be expressed as a weight/weight or weight/volume relationship. The conversion from a weight relationship to one of volume incorporates density as a factor. For dilute aqueous solutions, the density of the solvent is approximately equal to the density of the solution; thus, concentrations in mg/L are approximately equal to $10^{-3}$ g/$10^3$ g or parts per million (ppm); ones in µg/L are approximately equal to $10^{-6}$ g/$10^3$ g or parts per billion (ppb). In addition, concentration can be expressed in terms of molarity, normality, molality, and mole fraction. For example, to convert from weight/volume to molarity one incorporates molecular mass as a factor.

(ii) "Density" is the mass of a unit volume of a material. It is a function of temperature, hence the temperature at which it is measured should be specified. For a solid, it is the density of the impermeable portion rather than the bulk density. For solids and liquids, suitable units of measurement are g/$cm^3$. The density of a solution is the mass of a unit volume of the solution and suitable units of measurement are g/$cm^3$.

(iii) A "saturated solution" is a solution in which the dissolved solute is in equilibrium with an excess of undissolved solute; or a solution in equilibrium such that at a fixed temperature and pressure, the concentration of the solute in the solution is at its maximum value and will not change even in the presence of an excess of solute.

(iv) A "solution" is a homogeneous mixture of two or more substances constituting a single phase.

(v) A "generator column" is used to produce or generate saturated solutions of a solute in a solvent. The column (see Figure 1 under paragraph (b)(1)(i)(A) of this section) is packed with a solid support coated with the solute, i.e., the organic compound whose solubility is to be determined. When water (the solvent) is pumped through the column, saturated solutions of the solute are generated. Preparation of the generator column is described under paragraph (b)(1)(i) of this section.

(vi) An "extractor column" is used to extract the solute from the saturated solutions produced by the generator column. After extraction onto a chromatographic support, the solute is eluted with a solvent/water mixture and subsequently analyzed by high pressure liquid chromatography (HPLC). A detailed description of the preparation of the extractor column is given in paragraph (b)(1)(i) of this section.

(vii) The "sample loop" is a 1/16 in. O.D. (1.6 mm) stainless steel tube with an internal volume between 20 and 50 µL. The loop is attached to the sample injection valve of the HPLC and is used to inject standard solutions into the mobile phase of the HPLC when determining the response factor for the recording integrator. The exact volume of the loop must be determined as described in paragraph (b)(3)(i)(B)(*1*) of this section when the HPLC method is used.

(viii) The "response factor" (RF) is the solute concentration required to give a one unit area chromatographic peak or one unit output from the

HPLC recording integrator at a particular recorder attenuation. The factor is required to convert from units of area to units of concentration. The determination of the response factor is given in paragraph (b)(3)(i)(B)(*2*) of this section.

(3) *Principle of the test method.* (i) This test method is based on the dynamic coupled column liquid chromatographic (DCCLC) technique for determining the aqueous solubility of organic compounds that was initially developed by May et al. (1978) under paragraph (d)(2) and (3) of this section, modified by DeVoe et al. (1981) under paragraph (d)(1) of this section, and finalized by Wasik et al. (1981) under paragraph (d)(4) of this section. The DCCLC technique utilizes a generator column, extractor column and HPLC coupled or interconnected to provide a continuous closed flow system. Saturated aqueous solutions of the test compound are produced by pumping water through the generator column that is packed with a solid support coated with the compound. The compound is extracted from the saturated solution onto an extractor column, then eluted from the extractor column with a solvent/water mixture and subsequently analyzed by HPLC using an ultraviolet absorption detector operating at a suitable wavelength. Chromatogram peaks are recorded and integrated using a recording integrator. The concentration of the compound in the effluent from the generator column, i.e., the water solubility of the compound, is determined from the mass of the compound (solute) extracted from a measured volume of water (solvent).

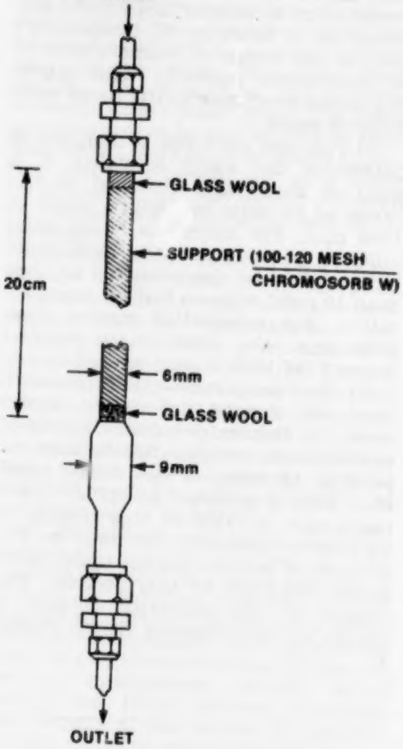
(ii) Since the HPLC method is only applicable to compounds that absorb in the ultraviolet, an alternate gas chromatographic (GC) method is used for those compounds that do not absorb in the ultraviolet. In the GC method the saturated solutions produced in the generator column are extracted using an appropriate organic solvent that is subsequently injected into the GC for analysis of the test compound.

(4) *Applicability and specificity.* (i) Procedures are described in this test guideline to determine the water solubility for liquid or solid compounds. The water solubility can be determined in very pure water, buffer solution for compounds that reversibly ionize or protonate, or in artificial seawater as a function of temperature (i.e., in the range of temperatures of environmental concern). This guideline is not applicable to the water solubility of gases.

(ii) This test guideline is designed to determine the water solubility of a solid or liquid test chemical in the range of 10 parts per billion (ppb) to 1000 ppm. For chemicals whose solubility is below 10 ppb, the water solubility should be characterized as "less than 10 ppb" with no further quantification. For solubilities greater than 1000 ppm, the shake flask method under § 796.1840 should be used.

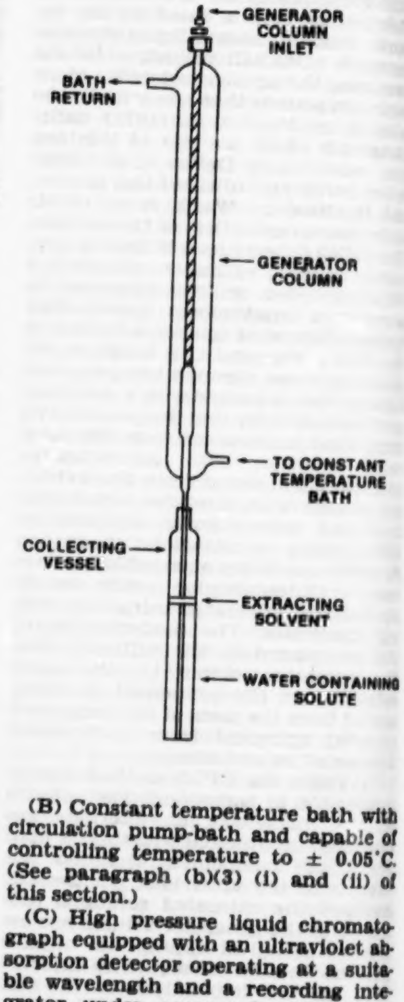
(b) *Test procedure*—(1) *Test conditions*—(i) *Special laboratory equipment.* (A) Generator column. Either of two different designs shall be used depending on whether the eluted aqueous phase is analyzed by HPLC under paragraph (b)(3)(i) of this section or by solvent extraction followed by GC analysis of solvent extract under paragraph (b)(3)(ii) of this section. The design of the generator column is shown in the following figure. Figure 1:

FIGURE 1—GENERATOR COLUMN


INLET
GLASS WOOL
SUPPORT (100-120 MESH CHROMOSORB W)
20cm
6mm
GLASS WOOL
9mm
OUTLET

The column consists of a 6 mm (¼-inch) O.D. Pyrex tube joined to a short enlarged section of 9 mm Pyrex tubing which in turn is connected to another section of 6 mm (¼-inch) O.D. Pyrex tubing. Connections to the inlet Teflon tubing (⅛-inch O.D.) and to the outlet stainless steel tubing ($^1/_{16}$-inch O.D.) shall be made by means of stainless steel fittings with Teflon ferrules. The column is enclosed in a water jacket for temperature control as shown in the following Figure 2:

FIGURE 2—SETUP SHOWING GENERATOR COLUMN ENCLOSED IN A WATER JACKET AND OVERALL ARRANGEMENT OF THE APPARATUS USED IN THE GC METHOD


GENERATOR COLUMN INLET
BATH RETURN
GENERATOR COLUMN
TO CONSTANT TEMPERATURE BATH
COLLECTING VESSEL
EXTRACTING SOLVENT
WATER CONTAINING SOLUTE

(B) Constant temperature bath with circulation pump-bath and capable of controlling temperature to ± 0.05°C. (See paragraph (b)(3) (i) and (ii) of this section.)

(C) High pressure liquid chromatograph equipped with an ultraviolet absorption detector operating at a suitable wavelength and a recording integrator under paragraph (b)(3)(i) of this section.

(D) Extractor column. 6.6 x 0.6 cm stainless steel tube with end fittings containing 5 μm frits filled with a superficially porous phase packing (Bondapack $C_{18}$/Corasil: Waters Associates) under paragraph (b)(3)(i) of this section.

(E) Two 6-port high pressure rotary switching valves under paragraph (b)(3)(i) of this section.

(F) Collection vessel. 8 x ¾ inch section of Pyrex tubing with a flat bottom connected to a short section of ⅜-inch O.D. borosilicate glass tubing in Figure 2 under paragraph (b)(1)(i)(A) of this section. The collecting vessel is sealed with a ⅜-inch Teflon cap fitting under paragraph (b)(3)(ii) of this section.

(G) Gas chromatograph equipped with a detector sensitive to the solute of interest under paragraph (b)(3)(ii) of this section.

(ii) *Purity of water.* Water meeting ASTM Type II standards or an equivalent grade, shall be used to minimize the effects of dissolved salts and other impurities on water solubility. ASTM Type II water is described in ASTM D 1193-77 (Reapproved 1983), "Standard Specification for Reagent Water." ASTM D 1193-77 (Reapproved 1983) is available for inspection at the Office of the Federal Register, Rm. 8401, 1100 L St., NW., Washington, DC. This incorporation by reference was approved by the Director of the Office of the Federal Register. This material is incorporated as it exists on the date of approval and a notice of any change in this material will be published in the FEDERAL REGISTER. Copies of the incorporated material may be obtained from the TSCA Public Docket Office (TS-793), Rm. NE-G004, Office of Toxic Substances, Environmental Protection Agency, 401 M St., Washington, DC 20460, or from the American Society for Testing and Materials (ASTM), 1916 Race Street, Philadelphia, PA 19103.

(iii) *Purity of solvents.* It is important that all solvents used in this method be reagent grade or better. Solvents shall contain no impurities which could interfere with the determination of the test compound.

(iv) *Seawater.* When the water solubility in seawater is desired, the artificial seawater described in paragraph (b)(2)(ii) of this section shall be used.

(v) *Effect of pH on solubility.* For chemicals that reversibly ionize or protonate with a $pK_a$ or $pK_b$ between 3 and 11, experiments shall be performed at pH 5.0, 7.0, and 9.0 using appropriate buffers.

(2) *Preparation of reagents and solutions*—(i) *Buffer solutions.* Prepare buffer solutions as follows:

(A) pH 5.0—To 250 mL of 0.1M potassium hydrogen phthalate add 113 mL of 0.1M sodium hydroxide; adjust the final volume to 500 mL with reagent grade water.

(B) pH 7.0—To 250 mL of 0.1M potassium dihydrogen phosphate add 145 mL of 0.1M sodium hydroxide; adjust the final volume to 500 mL with reagent grade water.

(C) pH 9.0—To 250 mL of 0.075M borax add 69 mL of 0.1M HCl; adjust the final volume to 500 mL with reagent grade water.

Check the pH of each buffer solution with a pH meter at 25 °C and adjust to pH 5.0, 7.0, or 9.0, if necessary. If the pH of the solution has changed by ±0.2 pH units or more after the addition of the test compound, then a more concentrated buffer is required for that pH determination. The sponsor should then choose a more suitable buffer.

(ii) *Artificial seawater.* Add the reagent-grade chemicals listed in the following Table 1 in the specified amounts and order to 890 mL of reagent-grade water. Each chemical shall be dissolved before another one is added.

TABLE 1—CONSTITUENTS OF ARTIFICIAL SEAWATER[1]

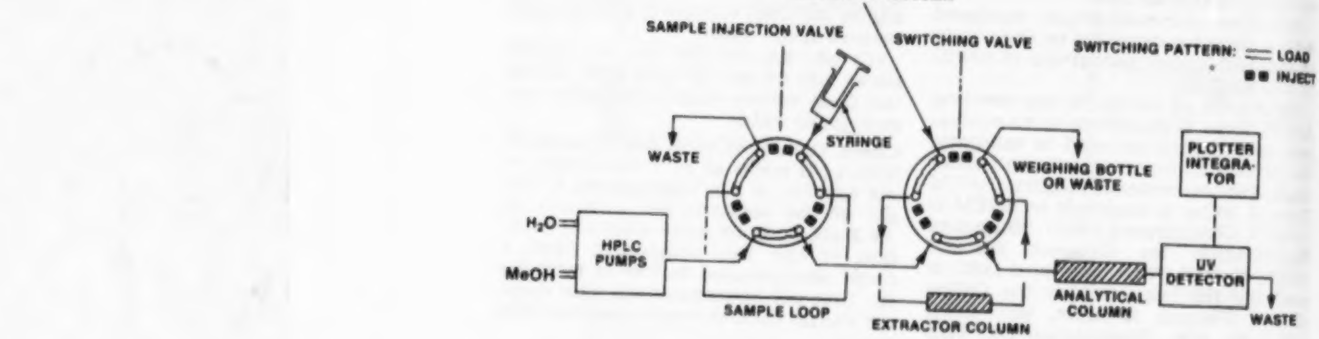
| Chemical | Amount |
|---|---|
| NaF | 3 mg |
| $SrCl_2 \cdot 6H_2O$ | 20 mg |
| $H_3BO_3$ | 30 mg |
| KBr | 100 mg |
| KCl | 700 mg |
| $CaCl_2 \cdot 2H_2O$ | 1.47 g |
| $Na_2SO_4$ | 4.00 g |
| $MgCl_2 \cdot 6H_2O$ | 10.78 g |
| NaCl | 23.50 g |
| $Na_2SiO_3 \cdot 9H_2O$ | 20 mg |
| $NaHCO_3$ | 200 mg |

[1] If the resulting solution is diluted to 1 cubic decimeter (1 liter), the salinity should be 34 ±0.5 g/kg and the pH 8.0 ±0.2. The desired test salinity is attained by dilution at time of use.

(3) *Performance of the test.* Using either the procedures under paragraph (b)(3) (i) or (ii) of this section, determine the water solubility of the test compound at 25 °C in reagent grade water or buffer solution, as appropriate. Under certain circumstances, it may be necessary to determine the water solubility of a test compound at 25 °C in artificial seawater. The water solubility can also be determined at other temperatures of environmental concern by adjusting the temperature of the water bath to the appropriate temperature.

(i) *Procedure A—HPLC Method*—(A) *Scope.* (*1*) Procedure A covers the determination of the aqueous solubility of compounds which absorb in the ultraviolet. The HPLC analytical system is shown schematically in the following Figure 3:

FIGURE 3—SCHEMATIC OF HPLC—GENERATOR COLUMN FLOW SYSTEM



Two reciprocating piston pumps deliver the mobile phase (water or solvent/water mixture) through two 6-port high pressure rotary valves and a 30 x 0.6 cm $C_{18}$/Corosil analytical column to an ultraviolet absorption detector operating at a suitable wavelength; chromatogram peaks are recorded and integrated with a recording integrator. One of the 6-port valves is the sample injection valve used for injecting samples of standard solutions of the solute in an appropriate concentration for determining response factors or standard solutions of basic chromate for determining the sample loop volume. The other 6-port valve in the system serves as a switching valve for the extractor column which is used to remove solute from the aqueous solutions.

(*2*) The general procedure for analyzing the aqueous phase is as follows: A detailed procedure is given in paragraph (b)(3)(i)(B)(*4*) of this section:

(*i*) Direct the aqueous solution to "Waste" (see Figure 3 under paragraph (b)(3)(i)(A)(*1*) of this section) with the switching valve in the inject position in order to equilibrate internal surfaces with the solution, thus insuring that the analyzed sample would not be depleted by solute adsorption on surfaces upstream from the valve.

(*ii*) At the same time, water is pumped from the HPLC pumps in order to displace the solvent from the extractor column.

(*iii*) The switching valve is next changed to the load position to divert a sample of the solution through the extractor column, and the liquid leaving this column is collected in a weighing bottle. During this extraction step, the mobile phase is changed to a solvent/water mixture to condition the analytical column.

(*iv*) After the desired volume of sample is extracted, the switching valve is returned to the inject position for elution and analysis. Provided that there is no breakthrough of solute from the extractor column during the extraction step, the chromatographic peak represents all of the solute in the sample.

(*v*) The solute concentration in the aqueous phase in calculated from the peak area and the weight of the extracted liquid collected in the weighing bottle.

(B) *Determinations—(1) Sample Loop Volume.* Accurate measurement of the sample loop may be accomplished by using the spectrophotometric method of Devoe et al. (1981) under paragraph (d)(1) of this section. For this method measure absorbance, $A_{loop}$, at 373 nm of at least three solutions, each of which is prepared by collecting from the sample valve an appropriate number, n, of loopfuls of an aqueous stock solution of $K_2CrO_4$ (1.3 percent by weight) and diluting to 50 mL with 0.2 percent KOH. (For a 20 μL loop, use n=5; for a 50 μL loop, use n=2.) Also measure the absorbance, $A_{stock}$, of the same stock solution after diluting 1:500 with 0.2 percent KOH. Calculate the loop volume to the nearest 0.1 μL using the relation:

$$V_{loop}=(A_{loop}/A_{stock})(10^{-4}/n)$$

(*2*) *Response Factor* (*RF*). (*i*) For all determinations adjust the mobile phase solvent/water ratio and flow rate to obtain a reasonable retention time on the HPLC column. For example, typical concentrations of solvent in the mobile phase range from 50 to 100 percent while flow rates range from 1 to 3 mL/min; these conditions give a 3 to 5 minute retention time.

(*ii*) Prepare standard solutions of known concentrations of the solute in a suitable solvent. Concentrations must give a recorder response within the maximum response of the detector. Inject samples of each standard solution into the HPLC system using the calibrated sample loop. Obtain an average peak area from at least three injections of each standard sample at a set absorbance unit full scale (AUFS), i.e., at the same absorbance scale attenuation setting.

(*iii*) Calculate the response factor from the following equation:

$$\text{Response Factor (RF)}=\frac{\text{Concentration (M)}}{\text{(Average Area) (AUFS)}}$$

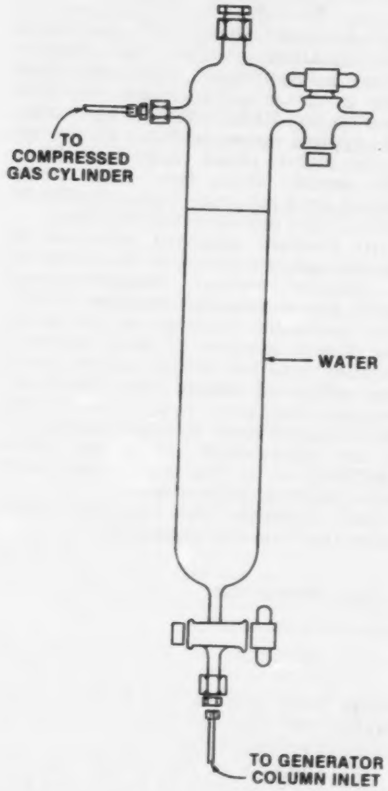
(*3*) *Loading of the Generator Column.* (*i*) The design of the generator column was described in paragraph (b)(1)(i) of this section and is shown in Figure 1 under paragraph (b)(1)(i)(A) of this section. To pack the column, a plug of silanized glass wool is inserted into one end of the 6 mm Pyrex tubing. Silanized diatomaceous silica support (about 0.5g 100–120 mesh Chromosorb W chromatographic support material) is poured into the tube with tapping and retained with a second plug of silanized glass wool.

(*ii*) If the solute is a liquid, the column is loaded by pulling the liquid solute through the dry support with gentle suction. If the solute is a solid, a 1 percent solution of the solid in a volatile solvent is added to the dry packing. The solvent is then distilled off the column under reduced pressure. After loading the column draw water up through the column to remove entrapped air.

(*4*) *Analysis of the solute.* Use the following procedure to collect and analyze the solute.

(*i*) Pump water to the generator column by means of a minipump or pressurized water reservoir as shown in the following Figure 4:

FIGURE 4—WATER RESERVOIR FOR GC METHOD



With the switching valve (Figure 3 under paragraph (b)(3)(i)(A)(*1*) of this section) in the inject position (i.e., water to waste), pump water through the generator column at a flow rate of approximately 1 mL/min for approximately 5 minutes to bring the system into equilibrium.

(*ii*) Flush out the solvent that remains in the system from previous runs by changing the mobile phase to 100 percent $H_2O$ and allowing the water to reach the HPLC detector, as indicated by a negative reading. As soon as this occurs, place a 25 mL weighing bottle (weighed to the nearest mg) at the waste position and immediately turn the switching valve to the load position.

(*iii*) Collect an amount of water (as determined by trial and error) in the weighing bottle, corresponding to the amount of solute adsorbed by the extractor column that gives a large on-scale detector response. During this extraction step, switch back to the original HPLC mobile phase composition, i.e., solvent/water mixture, to condition the HPLC analytical column.

(*iv*) After the desired volume of sample has been extracted, turn the switching valve back to the inject position (Figure 3 under paragraph (b)(3)(i)(A)(*1*) of this section); at the same time turn on the recording integrator. The solvent/water mobile phase will elute the solute from the extractor column and transfer the solute to the HPLC analytical column.

(*v*) Remove the weighing bottle, cap it and replace it with the waste container. Determine the weight of water collected to the nearest mg and record the corresponding peak area. Using the same AUFS setting repeat the analysis of the solute at least two more times and determine the average ratio of peak area to grams of water collected. Calculate the solute solubility in water using the following equation:

$$s=(997\ g/L)(RF)(V_{loop})(AUFS)(R)$$

where

$s$=solubility (M)
RF=response factor
$V_{loop}$=sample loop volume (L)
R=ratio of area to grams of water.

(ii) *Procedure B-GC Method.* (A) *Scope.* In the GC method, aqueous solutions from the generator column enter a collecting vessel (Figure 2 under paragraph (b)(1)(i)(A) of this section) containing a known weight of extracting solvent which is immiscible in water. The outlet of the generator column is positioned such that the aqueous phase always enters below the extracting solvent. After the aqueous phase is collected, the collecting vessel is stoppered and the quantity of aqueous phase is determined by weighing. The solvent and the aqueous phase are equilibrated by slowly rotating the collecting vessel. A small amount of the extracting solvent is then removed and injected into a gas chromatograph equipped with an appropriate detector. The solute concentration in the aqueous phase is determined from a calibration curve constructed using known concentrations of the solute.

(B) *Determinations—(1) Calibration curve.* (*i*) Prepare solute standard solutions of concentrations covering the range of the solute solubility. Select a column and optimum GC operating conditions for resolution between the solute and solvent and the solute and extracting solvent. Inject a known volume of each standard solution into the injection port of the GC. For each standard solution determine the average of the ratio R of peak area to volume (in μL) for three chromatographic peaks from three injections.

(*ii*) After running all the standard solutions, determine the coefficients, a and b, using a linear regression equation of concentration (c) vs. R in the following form

$$c = aR + b.$$

(*2*) *Loading of the generator column.* The generator column is packed and loaded with solute in the same manner as for the HPLC method (see paragraph (b)(3)(i)(B)(*3*) of this section). As shown in Figure 2 under paragraph (b)(1)(i)(A) of this section, attach approximately 20 cm of straight stainless steel tubing to the bottom of the generator column. Connect the top of the generator column to a water reservoir (Figure 4 under paragraph (b)(3)(i)(B)(*4*)(*i*) of this section) using Teflon tubing. Use air or nitrogen pressure (5 PSI) from an air or nitrogen cylinder to force water from the reservoir through the column. Collect water in an Erlenmeyer flask for approximately 15 minutes while the solute concentration in water equilibrates; longer time may be required for less soluble compounds.

(*3*) *Collection and extraction of the solute.* During the equilibration time, add a known weight of extracting solvent to a collection vessel which can be capped. The extracting solvent should cover the bottom of the collection vessel to a depth sufficient to submerge the collecting tube but still maintain 100:1 water/solvent ratio. Record the weight (to the nearest mg) of a collection vessel with cap and extracting solvent. Place the collection vessel under the generator column so that water from the collecting tube enters below the level of the extracting solvent (Figure 2 under paragraph (b)(1)(i)(A) of this section). When the collection vessel is filled, remove it from under the generator column, replace cap, and weigh the filled vessel. Determine the weight of water collected. Before analyzing for the solute, gently shake the collection vessel contents for approximately 30 min., controlling the rate of shaking so as not to form an emulsion; rotating the flask end over end five times per minute is sufficient.

(*4*) *Analysis of the solute.* (*i*) After shaking, allow the collection vessel to stand for approximately 30 minutes; then remove a known volume of the extracting solvent from the vessel using a microliter syringe and inject it into the G.C. Record the ratio of peak area to volume injected and, from the regression equation of the calibration line, determine the concentration of solute in the extracting solvent. The concentration of solute in water C(M) is determined from the following equation:

$$C(M) = (C_{es}) \frac{d_{H_2O}}{d_{es}} \frac{g_{es}}{g_{H_2O}}$$

where $C_{es}$ is the concentration of solute in extracting solvent (M), $d_{H_2O}$ and $d_{es}$ are the densities of water and extracting solvent, respectively, and $g_{es}$ and $g_{H_2O}$ are the grams of extracting solvent and water, respectively, contained in the collection vessel.

(*ii*) Make replicate injections from each collecting vessel to determine the average solute concentration in water for each vessel. To make sure the generator column has reached equilibrium, run at least two additional (for a total of three) collection vessels and analyze the extracted solute as described above. Calculate the water solubility of the solute from the average solute concentration in the three vessels.

(iii) *Modification of procedures for potential problems*—(A) *Decomposition of the test compound.* If the test compound decomposes in one or more of the aqueous solvents required during the period of the test at a rate such that an accurate value for water solubility cannot be obtained, then it will be necessary to carry out detailed transformation studies; e.g., hydrolysis under § 796.3500. If decomposition is due to aqueous photolysis, then it will be necessary to carry out water solubility studies in the dark, under red or yellow lights, or by any other suitable method to eliminate this transformation process.

(B) [Reserved]

(c) *Data and reporting*—(1) *Test report.* (i) For each set of conditions, (e.g., temperature, pure water, buffer solution, artificial seawater) required for the study, provide the water solubility value for each of three determinations, the mean value, and the standard deviation.

(ii) For compounds that decompose at a rate such that a precise value for the water solubility cannot be obtained, provide a statement to that effect.

(iii) For compounds with water solubility below 10 ppb, report the value as "less than 10 ppb".

(2) *Specific analytical, calibration and recovery procedures.* (i) For the HPLC method describe and/or report:

(A) The method used to determine the sample loop volume and the average and standard deviation of that volume.

(B) The average and standard deviation of the response factor.

(C) Any changes made or problems encountered in the test procedure.

(ii) For the GC method report:

(A) The column and GC operating conditions of temperature and flow rate.

(B) The average and standard deviation of the average area/$\mu$L obtained for each of the standard solutions.

(C) The form of the regression equation obtained in the calibration procedure.

(D) The extracting solvent used.

(E) The average and standard deviation of solute concentration in each collection vessel.

(F) Any changes made or problems encountered in the test procedure.

(d) *References.* For additional background information on this test guideline the following references should be consulted:

(1) DeVoe, H., Miller, M.M., Wasik, S.P. "Generator columns and high pressure liquid chromatography for determining aqueous solubilities and octanol-water partition coefficients of hydrophobic substances," *Journal of Research, National Bureau of Standards,* 86:361-366 (1981).

(2) May, W.E., Wasik, S.P., Freeman, D.H. Determination of the Aqueous Solubility of Polynuclear Aromatic Hydrocarbons by a Coupled Column Liquid Chromatographic Technique, *Analytical Chemistry.* 50:175-179.

(3) May, W.E., Wasik, S.P., Freeman, D.H. "Determination of the solubility behavior of some polycyclic aromatic hydrocarbons in the water," *Analytical Chemistry,* 50:997-1000 (1978).

(4) Wasik, S.P., Tewari, Y.B., Miller, M.M., Martire, D.E. "Octanol/Water Partition Coefficient and Aqueous Solubilities of Organic Compounds," NBS Report NBSIR 81-2406. Washington, DC: National Bureau of Standards, U.S. Department of Commerce, 1981.

[50 FR 39252, Sept. 27, 1985, as amended at 52 FR 19058, May 20, 1987; 53 FR 10391, Mar. 31, 1988; 53 FR 12525, Apr. 15, 1988]

### § 796.1950 Vapor pressure.

(a) *Introduction*—(1) *Background and purpose.* (i) Volatilization, the evaporative loss of a chemical, depends upon the vapor pressure of chemical and on environmental conditions which influence diffusion from a surface. Volatilization is an important source of material for airborne transport and may lead to the distribution of a chemical over wide areas and into bodies of water far from the site of release. Vapor pressure values provide indications of the tendency of pure substances to vaporize in an unperturbed situation, and thus provide a method for ranking the relative volatilities of chemicals. Vapor pressure data combined with water solubility data permit the calculation of Henry's law constant, a parameter essential to the calculation of volatility from water.

(ii) Chemicals with relatively low vapor pressures, high adsorptivity onto solids, or high solubility in water are less likely to vaporize and become airborne than chemicals with high vapor pressures or with low water solubility or low adsorptivity to solids and sediments. In addition, chemicals that are likely to be gases at ambient temperatures and which have low water solubility and low adsorptive tendencies are less likely to transport and persist in soils and water. Such chemicals are less likely to biodegrade or hydrolyze and are prime candidates for atmospheric oxidation and photolysis (e.g., smog formation or stratospheric alterations). On the other hand, nonvolatile chemicals are less frequently involved in atmosphere transport, so that concerns regarding them should focus on soils and water.

(iii) Vapor pressure data are an important consideration in the design of other chemical fate and effects tests; for example, in preventing or accounting for the loss of violatile chemicals during the course of the test.

(2) *Definitions and units.* (i) "Desorption efficiency" of a particular compound applied to a sorbent and subsequently extracted with a solvent s the weight of the compound which can be recovered from the sorbent divided by the weight of the compound originally sorbed.

(ii) "Pascal" (Pa) is the standard international unit of vapor pressure nd is defined as newtons per square meter ($N/m^2$). A newton is the force ecessary to give acceleration of one meter per second squared to one kiloram of mass.

(iii) The "torr" is a unit of pressure which equals 133.3 pascals or 1 mm Hg t 0 °C.

(iv) "Vapor pressure" is the pressure t which a liquid or solid is in equilibrium with its vapor at a given temperature.

(v) "Volatilization" is the loss of a substance to the air from a surface or from solution by evaporation.

(3) *Principle of the test methods.* (i) The isoteniscope procedure uses a standardized technique [ASTM 1978] that was developed to measure the vapor pressure of certain liquid hydrocarbons. The sample is purified within the equipment by removing dissolved and entrained gases until the measured vapor pressure is constant, a process called "degassing." Impurities more volatile than the sample will tend to increase the observed vapor pressure and thus must be minimized or removed. Results are subject to only slight error for samples containing nonvolatile impurities.

(ii) Gas saturation (or transpiration) procedures use a current of inert gas passed through or over the test material slowly enough to ensure saturation and subsequent analysis of either the loss of material or the amount (and sometimes kind) of vapor generated. Gas saturation procedures have been described by Spencer and Cliath (1969) under paragraph (d)(2) of this section. Results are easy to obtain and can be quite precise. The same procedures also can be used to study volatilization from laboratory scale environmental simulations. Vapor pressure is computed on the assumption that the total pressure of a mixture of gases is equal to the sum of the pressures of the separate or component gases and that the ideal gas law is obeyed. The partial pressure of the vapor under study can be calculated from the total gas volume and the weight of the material vaporized. If v is the volume which contains w grams of the vaporized material having a molecular weight M, and if p is the pressure of the vapor in equilibrium at temperature T (K), then the vapor pressure, p, of the sample is calculated by

$$p=(w/M)(RT/v),$$

where R is the gas constant (8.31 Pa $m^3mol^{-1} K^{-1}$) when the pressure is in pascals (Pa) and the volume is in cubic meters. As noted by Spencer and Cliath (1970) under paragraph (d)(3) of this section, direct vapor pressure measurements by gas saturation techniques are more directly related to the volatilization of chemicals than are other techniques.

(iii) In an effort to improve upon the procedure described by Spencer and Cliath (1969) under paragraph (d)(2) of this section, and to determine the applicability of the gas saturation method to a wide variety of chemical types and structures, EPA has sponsored research and development work at SRI International (EPA 1982) under paragraph (d)(1) of this section. The procedures described in this Test Guideline are those developed under that contract and have been evaluated with a wide variety of chemicals of differing structure and vapor pressures.

(4) *Applicability and specificity.* (i) A procedure for measuring the vapor pressure of materials released to the environment ideally would cover a wide range of vapor pressure values, at ambient temperatures. No single procedure can cover this range, so two different procedures are described in this section, each suited for a different part of the range. The isoteniscope procedure is for pure liquids with vapor pressures from 0.1 to 100 kPa. For vapor pressures of $10^{-5}$ to $10^3$ Pa, a gas saturation procedure is to be used.

(ii) With respect to the isoteniscope method, if compounds that boil close to or form azeotropes with the test material are present, it is necessary to remove the interfering compounds and use pure test material. Impurities more volatile than the sample will tend to increase the observed vapor pressure above its true value but the purification steps will tend to remove these impurities. Soluble, nonvolatile impurities will decrease the apparent vapor pressure. However, because the isoteniscope procedure is a static, fixed-volume method in which an insignificant fraction of the liquid sample is vaporized, it is subject to only slight error for samples containing nonvolatile impurities. That is, the nonvolatile impurities will not be concentrated due to vaporization of the sample.

(iii) The gas saturation method is applicable to solid or liquid chemicals. Since the vapor pressure measurements are made at ambient temperatures, the need to extrapolate data from high temperatures is not necessary and high temperature extrapolation, which can often cause serious errors, is avoided. The method is most reliable for vapor pressures below $10^3$ Pa. Above this limit, the vapor pressures are generally overestimated, probably due to aerosol formation. Finally, the gas saturation method is applicable to the determination of the vapor pressure of impure materials.

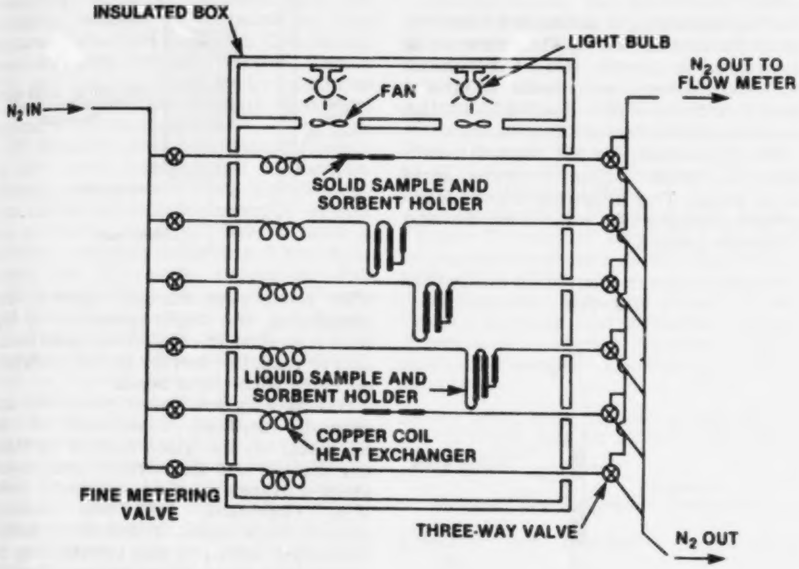
(b) *Test Procedures*—(1) *Test conditions.* (i) The apparatus in the isoteniscope method is described in paragraph (b)(2)(i) of this section.

(ii) The apparatus used in the gas saturation method is described in paragraph (b)(2)(ii) of this section.

(2) *Performance of the tests*—(i) *Isoteniscope Procedure.* The isoteniscope procedure described as ANSI/ASTM Method D 2879-86 is applicable for the measurement of vapor pressures of liquids with vapor pressures of 0.1 to 100 kilopascals (kPa) (0.75 to 750 torr). ASTM D 2879-86 is available for inspection at the Office of the Federal Register, Rm. 8301, 1100 L St., NW., Washington, DC. This incorporation by reference was approved by the Director of the Office of the Federal Register. This material is incorporated as it exists on the date of approval and a notice of any change in this material will be published in the FEDERAL REGISTER. Copies of the incorporated material may be obtained from the TSCA Public Docket Office (TS-793), Rm. NE-G004, Office of Toxic Substances, Environmental Protection Agency, 401 M St., SW., Washington, DC 20460, or from the American Society for Testing and Materials (ASTM), 1916 Race Street, Philadelphia, PA 19103. The isoteniscope method involves placing liquid sample in a thermostated bulb (the isoteniscope) connected to a manometer and a vacuum pump. Dissolved and entrained gases are removed from the sample in the isoteniscope by degassing the sample at reduced presssure. The vapor pressure of the sample at selected temperatures is determined by balancing the pressure due to the vapor of the sample against a known pressure of an inert gas. The vapor pressure of the test compound is determined in triplicate at 25±0.5 °C and at any other suitable temperatures (±0.5°). It is important that additional vapor pressure measurements be made at other temperatures, as necessary, to assure that there is no need for further degassing, as described in the ASTM method.

(ii) *Gas saturation procedure.* (A) The test procedures require the use of a constant-temperature box as depicted in the following Figure 1.

FIGURE 1—SCHEMATIC DIAGRAM OF VAPOR SATURATION APPARATUS


INSULATED BOX
LIGHT BULB
$N_2$ OUT TO FLOW METER
FAN
$N_2$ IN
SOLID SAMPLE AND SORBENT HOLDER
LIQUID SAMPLE AND SORBENT HOLDER
COPPER COIL HEAT EXCHANGER
FINE METERING VALVE
THREE-WAY VALVE
$N_2$ OUT

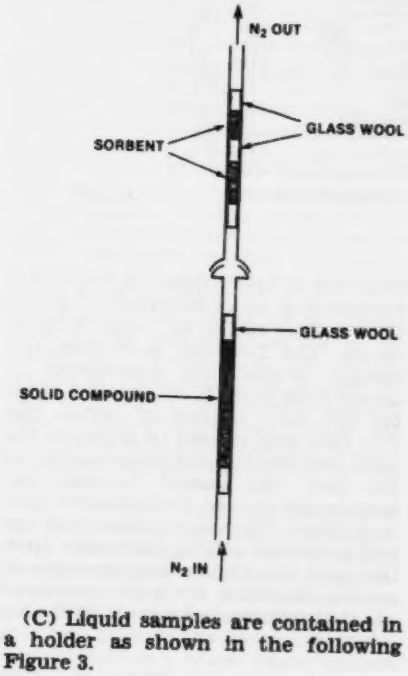
The insulated box, containing sample holders, may be of any suitable size and shape. The sketch in Figure 1 shows a box containing three solid sample holders and three liquid sample holders, which allows for the triplicate analysis of either a solid or liquid sample. The temperature within the box is controlled to ±0.5° or better. Nitrogen gas, split into six streams and controlled by fine needle valves (approximately 0.79 mm orifice), flows into the box via 3.8 mm 0.125 in.) i.d. copper tubing. After temperature equilibration, the gas flows through the sample and the sorbent trap and exits from the box. The flow rate of the effluent carrier gas is measured at room temperature with a bubble flow meter or other suitable device. The flow rate is checked frequently during the experiment to assure that there is an accurate value for the total volume of carrier gas. The flow rate is used to calculate the total volume (at room temperature) of gas that has passed through the sample and sorbent [(vol/time) × time = volume]. The vapor pressure of the test substance can be calculated from the total gas volume and the mass of sample vaporized. If v is the volume of gas that transported mass w of the vaporized test material having a molecular weight M, and if p is the equilibrium vapor pressure of the sample at temperature T, then p is calculated by the equation

$$p=(w/M)(RT/v).$$

In this equation, R is the gas constant ($8.31\ \mathrm{Pa\ m^3 mol^{-1}\ K^{-1}}$). The pressure is expressed in pascals (Pa), the volume in cubic meters ($m^3$), mass in grams and T in kelvins (K). $T=273.15+t$, if t is measured in degrees Celsius (°C).

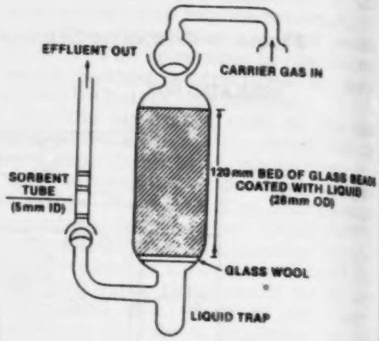
(B) Solid samples are loaded into 5 mm i.d. glass tubing between glass wool plugs. The following Figure 2 depicts a drawing of a sample holder and absorber system.

FIGURE 2—SOLID COMPOUND SAMPLING SYSTEM



(C) Liquid samples are contained in a holder as shown in the following Figure 3.

FIGURE 3—LIQUID COMPOUND SAMPLING SYSTEM



The most reproducible method for measuring the vapor pressure of liquids is to coat the liquid on glass beads and to pack the holder in the designated place with these beads.

(D) At very low vapor pressures and sorbent loadings, adsorption of the chemical on the glass wool separating the sample and the sorbent and on the glass surfaces may be a serious problem. Therefore, very low loadings should be avoided whenever possible. Incoming nitrogen gas (containing no interfering impurities) passes through a coarse frit and bubbles through a 38 cm column of liquid sample. The stream passes through a glass wool column to trap aerosols and then through a sorbent tube, as described above. The pressure drop across the glass wool column and the sorbent tube are negligible.

(E) With both solid and liquid samples, at the end of the sampling time, the front and backup sorbent sections are analyzed separately. The compound on each section is desorbed by adding the sorbent from that section to 1.0 ml of desorption solvent in a small vial and allowing the mixture to stand at a suitable temperature until no more test compound desorbs. It is extremely important that the desorption solvent contain no impurities which would interfere with the analytical method of choice. The resulting solutions are analyzed quantitatively by a suitable analytical method to determine the weight of sample desorbed from each section. The choice of the analytical method, sorbent, and desorption solvent is dictated by the nature of the test material. Commonly used sorbents include charcoal, Tenax GC, and XAD-2. Describe in detail the sorbent, desorption solvent, and analytical methods employed.

(F) Measure the desorption efficiency for every combination of sample, sorbent, and solvent used. The desorption efficiency is determined by injecting a known mass of sample onto a sorbent and later desorbing it and analyzing for the mass recovered. For each combination of sample, sorbent, and solvent used, carry out the determination in triplicate at each of three concentrations. Desorption efficiency may vary with the concentration of the actual sample and it is important to measure the efficiency at or near the concentration of sample under gas saturation test procedure conditions.

(G) To assure that the gas is indeed saturated with test compound vapor, sample each compound at three differing gas flow rates. Appropriate flow rates will depend on the test compound and test temperature. If the calculated vapor pressure shows no dependence on flow rate, then the gas is assumed to be saturated.

(c) *Data and reporting.* (1) Report the triplicate calculated vapor pressures for the test material at each temperature, the average calculated vapor pressure at each temperature, and the standard deviation.

(2) Provide a description of analytical methods used to analyze for the test material and all analytical results.

(3) For the isoteniscope procedure, include the plot of p vs. the reciprocal of the temperature in K, developed during the degasing step and showing linearity in the region of 298.15 K (25 °C) and any other required test temperatures.

(4) For the gas saturation procedure, include the data on the calculation of vapor pressure at three or more gas flow rates at each test temperature, showing no dependence on flow rate. Include a description of sorbents and solvents employed and the desorption efficiency calculations.

(5) Provide a description of any difficulties experienced or any other pertinent information.

(d) *References.* For additional background information on this test guideline the following references should be consulted:

(1) U.S. Environmental Protection Agency. *Evaluation of Gas Saturation Methods to Measure Vapor Pressures: Final Report,* EPA Contract No. 68-01-5117 with SRI International, Menlo Park, California (1982).

(2) Spencer, W.F. and Cliath, M.M. "Vapor Density of Dieldrin," *Journal of Agricultural and Food Chemistry,* 3:664-670 (1969).

(3) Spencer, W.F. and Cliath, M.M. "Vapor Density and Apparent Vapor Pressure of Lindane," *Journal of Agricultural and Food Chemistry,* 18:529-530 (1970).

[50 FR 39252, Sept. 27, 1985, as amended at 53 FR 12525, Apr. 15, 1988; 53 FR 21641, June 9, 1988]

## Subpart C—Transport Processes

### § 796.2700 Soil thin-layer chromatography.

(a) *Introduction*—(1) *Background and purpose.* (i) Leaching of chemicals through soil is an important process which affects a chemical's distribution in the environment. If a chemical is tightly adsorbed to soil particles, it will not leach through the soil profile but will remain on the soil surface. If a chemical is weakly adsorbed, it will leach through the soil profile and may reach ground waters and then surface waters. Knowledge of the leaching potential is essential under certain circumstances for the assessment of the fate of chemicals in the environment.

(ii) Chemical leaching also affects the assessment of ecological and human health effects of chemicals. If a chemical reaches ground water, deleterious human health effects may arise due to the consumption of drinking water. If a chemical remains at the soil surface, deleterious environmental and human health effects may arise due to an increased concentration of the chemical in the zone of plant growth, possibly resulting in contamination of human food supplies.

(iii) Soil thin-layer chromatography (TLC) is a qualitative screening tool suitable for obtaining an estimate of a chemical's leaching potential. This test is one of several tests which can be used in obtaining a rough estimation of a chemical's leaching potential.

(2) *Definitions and units.* (i) "Cation exchange capacity" (CEC) is the sum total of exchangeable cations that a soil can adsorb. The CEC is expressed in milliequivalents of negative charge per 100 grams (meq/100 g) or milliequivalents of negative charge per gram (meq/g) of soil.

(ii) "Particle size analysis" is the determination of the various amounts of the different particle sizes in a soil sample (i.e., sand, silt, clay) usually by sedimentation, sieving, micrometry, or combinations of these methods. The names and size limits of these particles as widely used in the United States are:

| Name | Diameter range |
|---|---|
| Very coarse sand | 2.0 to 1.0 mm |
| Coarse sand | 1.0 to 0.5 mm |
| Medium sand | 0.5 to 0.25 mm |
| Fine sand | 0.25 to 0.125 mm |
| Very fine sand | 0.125 to 0.062 mm |
| Silt | 0.062 to 0.002 mm |
| Clay | <0.002 mm |

(iii) "$R_f$" is the furthest distance traveled by a test material on a thin-layer chromatography plate divided by the distance traveled by a solvent front (arbitrarily set at 10.0 cm in soil TLC studies).

(iv) "Soil" is the unconsolidated mineral material on the immediate surface of the earth that serves as a natural medium for the growth of land plants; its formation and properties are determined by various factors such as parent material, climate, macro- and microorganisms, topography, and time.

(v) "Soil aggregate" is the combination or arrangement of soil separates (sand, silt, clay) into secondary units. These units may be arranged in the profile in a distinctive characteristic pattern that can be classified on the basis of size, shape, and degree of distinctness into classes, type, and grades.

(vi) "Soil classification" is the systematic arrangement of soils into groups or categories. Broad groupings are made on the basis of general characteristics, subdivisions, on the basis of more detailed differences in specific properties. The soil classification system used today in the United States is the 7th Approximation Comprehensive System. The ranking of subdivisions under the system is: order, suborder, great group, family and series.

(vii) "Soil horizon" is a layer of soil approximately parallel to the land surface. Adjacent layers differ in physical, chemical, and biological properties or characteristics such as color, structure, texture, consistency, kinds, and numbers of organisms present, and degree of acidity or alkalinity.

(viii) "Soil order" is the broadest category of soil classification and is based on general similarities of physical/chemical properties. The formation by similar genetic processes causes these similarities. The soil orders found in the United States are: Alfisol, Aridisol, Entisol, Histosol, Inceptisol, Mollisol, Oxisol, Spodosol, Ultisol, and Vertisol.

(ix) "Soil organic matter" is the organic fraction of the soil; it includes plant and animal residues at various stages of decomposition, cells and tissues of soil organisms, and substances synthesized by the microbial population.

(x) "Soil pH" is the negative logarithm to the base 10 of the hydrogen ion activity of a soil as determined by means of a suitable sensing electrode coupled with a suitable reference electrode at a 1:1 soil:water ratio.

(xi) "Soil series" is the basic unit of soil classification and is a subdivision of a family. A series consists of soils that were developed under comparable climatic and vegetational conditions. The soils comprising a series are essentially alike in all major profile characteristics except for the texture of the "A" horizon (i.e., the surface layer of soil).

(xii) "Soil texture" refers to the classification of soils based on the relative proportions of the various soil separates present. The soil textural classes

re: clay, sandy clay, silty clay, clay
am, silty clay loam, sandy clay loam,
am, silt loam, silt, sandy loam, loamy
nd, and sand.

(3) *Principle of the test method.* (i)
efore 1968, methods of investigating
e mobility of nonvolatile organic
emicals within soils were based on
e use of field analysis, soil adsorp-
on isotherms, and soil columns. In
68, Helling and Turner introduced
il thin-layer chromatography (soil
LC) as an alternative procedure; it is
alogous to conventional TLC, with
e use of soil instead of silica gels,
ides, etc., as the adsorbent phase.

(ii) The papers by Helling (1968,
71) under paragraphs (d) (5), (6),
d (7) of this section and Helling and
rner (1968) under paragraph (d)(3)
this section were the basis of this
t guideline. The soil and colloid
emistry literature and the analytical
emistry literature substantiate the
perimental conditions specified in
e guideline.

iii) The soil TLC offers many desir-
le features. First, mobility results
e reproducible. Mass transfer and
fusion components are distinguish-
le. The method has relatively
dest requirements for chemicals,
ls, laboratory space, and equipment.
yields data that are amenable to sta-
ical analyses. A chemical extrac-
n-mass balance procedure to elicit
ormation on degradation and chem-
l transformations occurring at col-
d interfaces can be incorporated
o this test. The ease with which the
and mass balance are performed
l depend upon the physical/chemi-
properties of the test chemical and
availability of suitable analytical
hniques for measuring the chemi-

) *Applicability and specificity.* (i)
l TLC can be used to determine the
mobility of sparingly water soluble
nfinitely soluble chemicals. In gen-
l, a chemical having a water solubil-
of less than 0.5 ppm need not be
ed since the literature indicates
t these chemicals are, in general,
obile, see Goring and Hamaker
72) under paragraph (d)(1) of this
ion. However, this does not pre-
le future soil adsorption/transfor-
ion testing of these chemicals if
more refined data are needed for the assessment process.

(ii) Soil TLC may be used to test the mobility of volatile chemicals by placing a clean plate over the spotted soil TLC plate and then placing both plates in a closed chromatographic chamber.

(iii) Soil TLC was originally designed for use with soils. The literature shows no published use of this method with sediments as the absorbent phase, probably due to the fact that sediment surface properties change significantly during air drying. It is extremely important that the TLC plate with the adsorbent be air dried before leaching studies can be undertaken.

(b) *Test procedures*—(1) *Test conditions.* (i) Equipment required: Distilled-deionized water adjusted to pH 7 by boiling to remove $CO_2$; clean glass plates (TLC); glass rods or a variable thickness plate spreader; masking tape; closed chromatographic chambers; analytical instrumentation necessary and appropriate for the detection and quantitative analysis of the test chemical.

(ii) The test procedure may be run at 23±5 °C.

(iii) It is recommended that three replicate plates for each soil be used.

(2) *Test procedures.* (i) To reduce aggregate size before or during sieving, crush and grind the air-dried soil very, very gently.

(ii) Sieve air-dried soils with a 250 micrometer sieve.

(iii) Add water to the sieved soil until a smooth, moderately fluid slurry is attained (approximately ¾ ml $H_2O$ added for each gram of soil).

(iv) Spread the slurry evenly and quickly across the clean glass plate using a variable thickness plate spreader, a glass rod, or other available method. If a glass rod is used, control the layer thickness by affixing multiple layers of masking tape along the plate edges. Soil layer thickness should be 0.50–0.75 mm.

(v) Air dry the plates at 25 °C for a minimum of 24 hours after uniform slurry application is achieved.

(vi) Scribe a horizontal line 11.5 cm above the base through the soil layer down to the glass so as to stop solvent movement.

(vii) Spot the test chemical, in solution, 1.5 cm above the base. For radiolabeled materials, 0.5-5 $\mu$g containing 0.01-0.03 $\mu$Ci of $^{14}$C labeled compound may be used.

(viii) If the compound is volatile, it is extremely important that a clean plate be placed over the soil TLC plate to impede volatilization.

(ix) Immerse the plate with the base down at some angle from the vertical in a closed chromatographic chamber containing $H_2O$ at a height of 0.5 cm.

(x) Allow the solvent front to migrate to the 11.5 cm line before removing the plates from the chamber.

(xi) Determine the $R_f$ values. Zonal extraction, plate scanning, or any other method or combination of methods suitable for detection of the parent test chemical may be used.

(xii) Determine the amount of the parent test chemical on the entire soil TLC plate after test chemical migration. Any method or combination of methods suitable for the extraction and quantitative detection of the parent test chemical may be used.

(c) *Data and reporting.* Report the following information. (1) Temperature at which the test was conducted.

(2) Amount of the test chemical applied and amount recovered from the plates.

(3) Detailed description of the analytical technique used in the $R_f$ determination, the chemical extraction, and the quantitative recovery and analysis of the parent chemical.

(4) The mean frontal $R_f$ value with the standard deviation for each soil tested.

(5) A photograph or diagram of the TLC plate which shows the entire leaching pattern (from 1.5 to 11.5 cm).

(6) Soil information: soil order, series, texture, sampling location, horizon, general clay fraction mineralogy.

(7) Soil physical/chemical properties: percent sand, percent silt, and percent clay (particle size analysis); percent organic matter; pH (soil-to-water ratio, 1:1); and cation exchange capacity.

(d) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Goring, C.A.I., Hamaker, J.W. *Organic Chemicals in the Soil Environment.* Vol. I & II (New York: Marcel Dekker, Inc., 1972).

(2) Helling, C.S. "Pesticide mobility investigations using soil thin-layer chromatography," *American Society for Agronomy Abstracts* (1968).

(3) Helling, C.S., Turner, B.C. "Pesticide mobility: Determination by soil thin layer chromatography," *Science,* 162:562 (1968).

(4) Helling, C.S., "Movement of s-triazine herbicides in soils," *Residue Review,* 32:175-210 (1970).

(5) Helling, C.S. "Pesticide mobility in soils I. Parameters of soil thin layer chromatography," *Soil Science Society of America Proceedings,* 35:732-737 (1971).

(6) Helling, C.S. "Pesticide mobility in soils II. Applications of soil thin layer chromatography," *Soil Science Society of America Proceedings,* 35:737-743 (1971).

(7) Helling, C.S. "Pesticide mobility in soils III. Influence of soil properties," *Soil Science of America Proceedings,* 35:743-748 (1971).

[50 FR 39252, Sept. 27, 1985]

### § 796.2750 Sediment and soil adsorption isotherm.

(a) *Introduction*—(1) *Background and purpose.* The adsorption of chemicals to sediments and soils is an important process that affects a chemical's distribution in the environment. If a chemical is adsorbed to soil particles, it will remain on the soil surface and will not reach ground water. If a chemical is not adsorbed, it will leach through the soil profile and may reach ground waters and then surface waters. Similarly, if a chemical adsorbed to sediment, it will accumulate in the bed and suspended load of aquatic systems. If a chemical is not adsorbed to sediment, it will accumulate in the water column of aquatic systems. Information on the adsorption potential is needed under certain circumstances to assess the transport of chemicals in the environment. This section describes procedures that will enable sponsors to determine the adsorption isotherm of a chemical on sediments and soils.

(2) *Definitions and units.* (i) The "cation exchange capacity" (CEC) is the sum total of exchangeable cations that a sediment or soil can adsorb. The CEC is expressed in milliequivalents of negative charge per 100 grams (meq/100g) or milliequivalents of negative charge per gram (meq/g) of soil or sediment.

(ii) "Clay mineral analysis" is the estimation or determination of the kinds of clay-size minerals and the amount present in a sediment or soil.

(iii) "Organic matter" is the organic fraction of the sediment or soil; it includes plant and animal residues at various stages of decomposition, cells and tissues of soil organisms, and substances synthesized by the microbial population.

(iv) "Particle size analysis" is the determination of the various amounts of the different particle sizes in a sample (i.e., sand, silt, clay), usually by sedimentation, sieving, micrometry, or combinations of these methods. The names and diameter range commonly used in the United States are:

| Name | Diameter range |
|---|---|
| ery coarse sand | 2.0 to 1.0 mm |
| oarse sand | 1.0 to 0.5 mm |
| edium sand | 0.5 to 0.25 mm |
| ine sand | 0.25 to 0.125 mm |
| ery fine sand | 0.125 to 0.062 mm |
| [illegible] | 0.062 to 0.002 mm |
| ay | <0.002 mm |

(v) The "pH" of a sediment or soil is he negative logarithm to the base ten f the hydrogen ion activity of the ediment or soil suspension. It is usualy measured by a suitable sensing elecrode coupled with a suitable refernce electrode at a 1/1 solid/solution atio by weight.

(vi) The adsorption ratio, "$K_d$," is he amount of test chemical adsorbed y a sediment or soil (i.e., the solid hase) divided by the amount of test hemical in the solution phase, which in equilibrium with the solid phase, a fixed solid/solution ratio.

(vii) "Sediment" is the unconsolidat- inorganic and organic material that suspended in and being transported surface water, or has settled out d has deposited into beds.

(viii) "Soil" is the unconsolidated mineral material on the immediate surface of the earth that serves as a natural medium for the growth of land plants. Its formation and properties are determined by various factors such as parent material, climate, macro- and microorganisms, topography, and time.

(ix) "Soil aggregate" is the combination or arrangement of soil separates (sand, silt, clay) into secondary units. These units may be arranged in the soil profile in a distinctive characteristic pattern that can be classified according to size, shape, and degree of distinctness into classes, types, and grades.

(x) "Soil classification" is the systematic arrangement of soils into groups or categories. Broad groupings are based on general soil characteristics while subdivisions are based on more detailed differences in specific properties. The soil classification system used in this standard and the one used today in the United States is the 7th Approximation-Comprehensive System. The ranking of subdivisions under this system is: Order, Suborder, Great group, family, and series.

(xi) A "soil horizon" is a layer of soil approximately parallel to the land surface. Adjacent layers differ in physical, chemical, and biological properties such as color, structure, texture, consistency, kinds and numbers of organisms present, and degree of acidity or alkalinity.

(xii) "Soil Order" is the broadest category of soil classification and is based on the general similarities of soil physical/chemical properties. The formation of soil by similar general genetic processes causes these similarities. The Soil Orders found in the United States are: Alfisol, Aridisol, Entisol, Histosol, Inceptisol, Mollisol, Oxisol, Spodosol, Ultisol, and Vertisol.

(xiii) "Soil series" is the basic unit of soil classification and is a subdivision of a family. A series consists of soils that were developed under comparable climatic and vegetational conditions. The soils comprising a series are essentially alike in all major profile characteristics except for the texture of the

"A" horizon (i.e., the surface layer of soil).

(xiv) "Soil texture" is a classification of soils that is based on the relative proportions of the various soil separates present. The soil textural classes are: clay, sandy clay, silty clay, clay loam, silty clay loam, sandy clay loam, loam, silt loam, silt, sandy loam, loamy sand, and sand.

(3) *Principle of the test method.* (i) The extent of adsorption of a chemical onto sediment or soil is measured, using this test guideline, by equilibrating aqueous solutions containing different, but environmentally realistic, concentrations of the test chemical with a known quantity of sediment or soil. After equilibrium is reached, the distribution of the chemical between the water phase and the solid phase is quantitatively measured by a suitable analytical method. Then, sorption constants are calculated by using the Freundlich equation:

*Equation 1*

$$x/m = C_s = KC_e^{1/n}$$

where:

$C_e$ = Equilibrium concentration of the chemical in the solution phase

$C_s$ = Equilibrium concentration of the chemical in the solid phase

K = Freundlich adsorption coefficient

m = The mass of the solid in grams

$^{1/n}$ = Exponent where n is a constant

x = The mass in micrograms of the chemical adsorbed by m grams of solid.

Logarithmetic transformation of the Freundlich equation yields the following linear relationship:

*Equation 2*

$$\log C_s = \log K + (1/n) \log C_e$$

(ii) In order to estimate the environmental movement of the test chemical, the values K and 1/n are compared with the values of other chemicals whose behavior in soil and sediment systems is well-documented in scientific literature.

(iii) The adsorption isotherm (AI) test has many desirable features. First, adsorption results are highly reproducible. The test provides excellent quantitative data readily amenable to statistical analyses. Also, it has relatively modest requirements for chemicals, soils, laboratory space, and equipment. It allows solution phase organic chemical determinations that are relatively uncomplicated. A chemical extraction-mass balance procedure to elicit information on chemical transformations occurring at colloid interfaces can be incorporated into this test. The ease of performing the isotherm test and mass balance will depend upon the physical/chemical properties of the test chemical and the availability of suitable analytical techniques to measure the chemical.

(iv) The papers by Aharonson and Kafkafi (1975) under paragraph (d)(1) of this section, Harvey (1974) under paragraph (d)(3) of this section, Murray (1975) under paragraph (d)(4) of this section, Saltzman (1972) under paragraph (d)(5) of this section, Weber (1971) under paragraph (d)(6) of this section, and Wu (1975) under paragraph (d)(7) of this section served as the basis for this section. The soil and colloid chemistry literature and the analytical chemistry literature substantiate the experimental conditions and procedures specified in this guideline as accepted, standard procedures.

(4) *Applicability and specificity.* The AI Test Guideline can be used to determine the soil and sediment adsorption potential of sparingly water soluble to infinitely soluble chemicals. In general, a chemical having a water solubility of less than 0.5 ppm need not be tested with soil as the solid phase, since the literature indicates that these chemicals are, in general, immobile in soils, see Goring and Hamaker (1972) under paragraph (d)(2) of this section. However, this does not preclude future soil adsorption/transformation testing of these chemicals if more refined data are needed for the assessment process.

(b) *Test procedures*—(1) *Test conditions*—(i) *Special laboratory equipment.* (A) Equilibrating solutions that contain, besides the test chemical, 0.01M calcium nitrate dissolved in sterilized, distilled-deionized $H_2O$ adjusted to neutral pH 7 by boiling to remove $CO_2$.

(B) Containers shall be composed of material that (*1*) adsorb negligible amounts of test chemical, and (*2*) withstand high speed centrifugation.

The volume of the container is not a major consideration; however, it is extremely important that the amount of soil or sediment and the solid/solution ratio used in the study result in minimal container headspace. It is also extremely important that the containers be sterilized before use.

(C) A 150 micron (100 mesh) stainless-steel or brass sieve.

(D) Drying oven, with circulating air, that can attain 100 °C.

(E) Vortex mixer or a comparable device.

(F) Rotary shaker or a comparable device.

(G) High speed temperature-controlled centrifuge capable of sedimenting particles greater than 0.5 micron from aqueous solution.

(ii) *Temperature.* The test procedure shall be performed at 23±5 °C.

(iii) *Replications.* Three replications of the experimental treatments shall be used.

(iv) *Soil pretreatment.* The following soil pretreatment steps shall be performed under the following conditions:

(A) Decrease the water content, air or oven-dry soils at or below 50 °C.

(B) Reduce aggregate size before and during sieving, crush and grind dried soil very gently.

(C) Eliminate microbial growth during the test period using a chemical or physical treatment that does not alter or minimally alters the soil surface properties.

(D) Sieve soils with a 100 mesh stainless-steel or brass sieve.

(E) Store all solutions and soils at temperatures between 0 and 5 °C.

(v) *Sediment pretreatment.* The following sediment pretreatment steps shall be performed under the following conditions:

(A) Decrease the $H_2O$ content by air or oven-drying sediments at or below 50 °C. Sediments should not be dried completely and should remain moist at all times prior to testing and analysis.

(B) Eliminate microbial growth during the test period by using a chemical and/or physical treatment that does not alter or minimally alters the colloid surface's properties.

(C) Store at temperatures between 0 and 5 °C.

(vi) *Solid/solution ratio.* The solid/solution ratio shall be equal to or greater than 1/10. If possible, the ratios should be equal to or greater than 1/5. The sediment or soil dry weight after drying for a 24-hour minimum at 90 °C is recommended for use as the weight of the solid for ratio and data calculations. If an insufficient amount of chemical remains in the water phase for quantification, the solid/solution ratio should be adjusted so that measurable amounts of the test chemical remain in solution.

(vii) *Equilibration time.* The equilibration time will depend upon the length of time needed for the parent chemical to attain an equilibrium distribution between the solid phase and the aqueous solution phase. The equilibration time shall be determined by the following procedure:

(A) Equilibrate one solution containing a known concentration of the test chemical with the sediment or soil in a solid/solution ratio not exceeding 1/10 and preferably equal to or greater than 1/5. It is important that the concentration of the test chemical in the equilibrating solution (*1*) does not exceed one-half of its solubility and (*2*) should be 10 ppm or less at the end of the equilibration period.

(B) Measure the concentration of the chemical in the solution phase at frequent intervals during the equilibration period.

(C) Determine the equilibration time by plotting the measured concentration versus time of sampling; the equilibration time is the minimum period of time needed to establish a rate of change of solution concentration of 5 percent or less per 24 hours.

(viii) *Centrifugation time.* Calculate the centrifugation time, $t_c$, necessary to remove particles from solution greater than approximately 0.5 μm ($5\times10^{-4}$ μm) equivalent diameter (which represents all particles except the fine clay fraction) using the following equation:

*Equation 3*

$$t_c(\text{min})=1.41\times10^{9}\,[\log(R_2/R_1)]/N^2$$

where:

$t_c$ = centrifuge time in minutes

$R_2$ = distance from centrifuge spindle to deposition surface of centrifuge

$R_t$=distance from spindle to surface of the sample

N=number of revolutions of the centrifuge per minute.

(ix) *Storage of solutions.* If the chemical analysis is delayed during the course of the experiment, store all solutions between 0 and 5 °C.

(x) *Solvents for extraction.* It is important that the solvent used to extract the chemical from the sediment or soil is reagent grade or better. Solvents shall contain no impurities which could interfere with the determination of the test compound.

(2) *Test procedure*—(i) *Equilibration.* Add six solutions containing different concentrations of the test chemical to at least one gram of each solid. The initial concentration of the test chemical in these solutions will depend on the affinity the chemical has for the sediment or soil. Therefore, after equilibrium is attained, it is extremely important that the highest concentration of the test chemical in the equilibrating solution does not exceed 10 ppm, is at least one order of magnitude greater than the lowest concentration reported, and does not exceed one half of its solubility.

(A) Immediately after the solutions are added to the solids, tightly cap the containers and vigorously agitate them for several minutes with a vortex mixture or similar device.

(B) Shake the containers throughout the equilibration period at a rate that suspends all solids in the solution phase.

(ii) *Centrifugation.* When the equilibration time has expired, centrifuge the containers for $t_c$ minutes.

(iii) *Chemical extraction.* (A) After centrifugation, remove the supernatant aqueous phase from the solid-solution mixture.

(B) Extract the chemical adsorbed on the sediment or soil colloid surfaces with solvent.

(iv) *Chemical analysis.* Determine the amount of parent test chemical in the aqueous equilibrating solution and organic solvent extractions. Use any method or combination of methods suitable for the identification and quantitative detection of the parent test chemical.

(c) *Reporting.* Report the following information:

(1) Temperature at which the test was conducted.

(2) Detailed description of the analytical technique(s) used in the chemical extraction, recovery, and quantitative analysis of the parent chemical.

(3) Amount of parent test chemical applied, the amount recovered, and the percent recovered.

(4) Extent of adsorption by containers and the approach used to correct the data for adsorption by containers.

(5) The individual observations, the mean values, and graphical plots of x/m as a function of $C_e$ for each sediment or soil for (i) the equilibration time determination and (ii) the isotherm determination.

(6) The quantities K, n, and 1/n.

(7) Soil information: Soil Order, series, texture, sampling location, horizon, general clay fraction mineralogy.

(8) Sediment information: sampling location, general clay fraction mineralogy.

(9) Sediment and soil physical-chemical properties: percent sand, silt, and clay (particle size analysis); percent organic matter; pH (1/1 solids/$H_2O$); and cation exchange capacity.

(10) The procedures used to determine the physical/chemical properties listed under paragraphs (c) (7) through (9) of this section.

(d) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Aharonson, N., Kafkafi, U. "Adsorption, mobility and persistence of thiabendazole and methyl 2-benzimidasole carbamate in soils," *Journal of Agricultural and Food Chemistry,* 23:720-724 (1975).

(2) Goring, C.A.I., Hamaker, J.W., (eds). *Organic Chemicals in the Soil Environment.* Vol. I & II (New York: Marcel Dekker, Inc., 1972).

(3) Harvey, R.G. et al. "Soil adsorption and volatility of dinitroaniline herbicides," *Weed Science,* 22:120-124 (1974).

(4) Murray, D.S. et al. "Comparative adsorption, desorption, and mobility of dipropetryn and prometryn in soil," *Journal of Agricultural and Food Chemistry,* 23:578-581 (1973).

(5) Saltzman, S.L. et al. "Adsorption, desorption of parathion as affected by soil organic matter," *Journal of Agricultural and Food Chemistry*, 20:1224-1226 (1972).

(6) Weber, J.B. "Model soil system, herbicide leaching, and sorption," *Weed Science*, 19:145-160 (1971).

(7) Wu, C.H., et al. "Napropamide adsorption, desorption, and movement in soils," *Weed Science*, 23:454-457 (1975).

[50 FR 39252, Sept. 27, 1985, as amended at 52 FR 19058, May 20, 1987]

## Subpart D—Transformation Processes

### § 796.3100 Aerobic aquatic biodegradation.

(a) *Introduction*—(1) *Purpose.* (i) This Guideline is designed to develop data on the rate and extent of aerobic biodegradation that might occur when chemical substances are released to aquatic environments. A high biodegradability result in this test provides evidence that the test substance will be biodegradable in natural aerobic freshwater environments.

(ii) On the contrary, a low biodegradation result may have other causes han poor biodegradability of the test ubstance. Inhibition of the microbial noculum by the test substance at the est concentration may be observed. In uch cases, further work is needed to ssess the aerobic aquatic biodegradaility and to determine the concentraions at which toxic effects are evient. An estimate of the expected enironmental concentration will help to ut toxic effects into perspective.

(2) *Definitions.* (i) "Adaptation" is he process by which a substance inuces the synthesis of any degradative nzymes necessary to catalyze the ransformation of that substance.

(ii) "Ultimate Biodegradability" is he breakdown of an organic comound to $CO_2$, water, the oxides or ineral salts of other elements and/or products associated with normal etabolic processes of microorgasms.

(iii) "Ready Biodegradability" is an pression used to describe those subances which, in certain biodegradaon test procedures, produce positive sults that are unequivocal and which ad to the reasonable assumption that the substance will undergo rapid and ultimate biodegradation in aerobic aquatic environments.

(3) *Principle of the test method.* This Guideline method is based on the method described by William Gledhill (1975) under paragraph (d)(1) of this section. The method consists of a 2-week inoculum buildup period during which soil and sewage microorganisms are provided the opportunity to adapt to the test compound. This inoculum is added to a specially equipped Erlenmeyer flask containing a defined medium with test substance. A reservoir holding barium hydroxide solution is suspended in the test flask. After inoculation, the test flasks are sparged with $CO_2$-free air, sealed, and incubated, with shaking in the dark. Periodically, samples of the test mixture containing water-soluble test substances are analyzed for dissolved organic carbon (DOC) and the $Ba(OH)_2$ from the reservoirs is titrated to measure the amount of $CO_2$ evolved. Differences in the extent of DOC disappearance and $CO_2$ evolution between control flasks containing no test substance, and flasks containing test substance are used to estimate the degree of ultimate biodegradation.

(4) *Prerequisites.* The total organic carbon (TOC) content of the test substance shall be calculated or, if this is not possible, analyzed, to enable the percent of theoretical yield of carbon dioxide and percent of DOC loss to be calculated.

(5) *Guideline information.* (i) Information on the relative proportions of the major components of the test substance will be useful in interpreting the results obtained, particularly in those cases where the result lies close to a "pass level."

(ii) Information on the toxicity of the chemical may be useful in the interpretation of low results and in the selection of appropriate test concentrations.

(6) *Reference substances.* Where investigating a chemical substance, reference compounds may be useful and an inventory of suitable reference compounds needs to be identified. In order to check the activity of the inoculum the use of a reference compound is desirable. Aniline, sodium cit-

rate, dextrose, phthalic acid and trimellitic acid will exhibit ultimate biodegradation under the conditions of this Test Guideline method. These reference substances must yield 60 percent of theoretical maximum $CO_2$ and show a removal of 70 percent DOC within 28 days. Otherwise the test is regarded as invalid and shall be repeated using an inoculum from a different source.

(7) *Reproducibility.* The reproducibility of the method has not yet been determined; however it is believed to be appropriate for a screening test which has solely an acceptance but no rejective function.

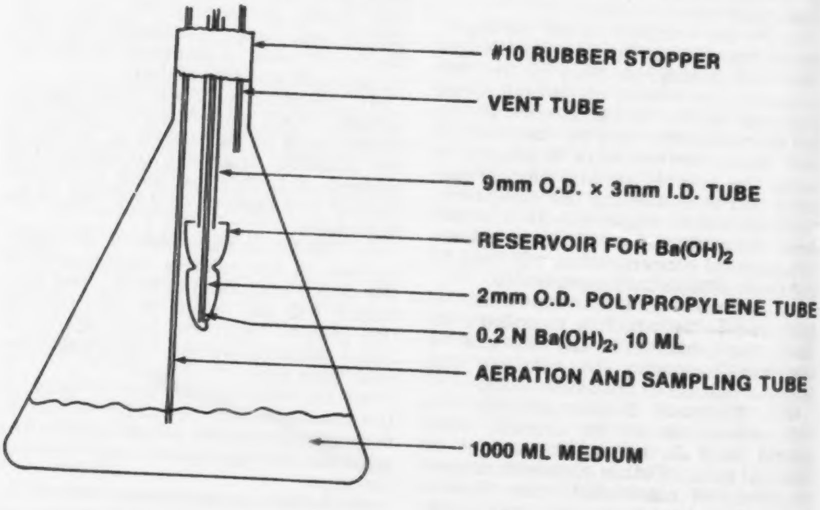
(8) *Sensitivity.* The sensitivity of the method is determined by the ability to measure the endogenous $CO_2$ production of the inoculum in the blank flask and by the sensitivity limit of the dissolved organic carbon analysis. If the test is adapted to handle $^{14}$C-labeled test substances, test substance concentrations can be much lower.

(9) *Possibility of standardization.* This possibility exists. The major difficulty is to standardize the inoculum in such a way that interlaboratory reproducibility is ensured.

(10) *Possibility of automation.* None at present, although parts of the analyses may be automated.

(b) *Test procedures*—(1) *Preparations*—(i) *Apparatus.* The shake flask apparatus under the following Figure 1 contains 10 mL of 0.2N $Ba(OH)_2$ in an open container suspended over 1 liter of culture medium in a 2-liter Erlenmeyer flask.

FIGURE 1—SHAKE-FLASK SYSTEM FOR CARBON DIOXIDE EVOLUTION



The $Ba(OH)_2$ container is made by placing a constriction just above the 10 mL mark of a 50 mL heavy-duty centrifuge tube and attaching the centrifuge tube to a 2 mm I.D. × 9 mm O.D. glass tube by means of 3 glass support rods. The centrifuge tube opening is large enough to permit $CO_2$ to diffuse into the $Ba(OH)_2$, while the constriction permits transferal of the flask to and from the shaker without $Ba(OH)_2$ spillage into the medium. For periodic removal and addition of base from the center well, a polypropylene capillary tube, attached at one end to a 10 ml disposable syringe, is inserted through the 9 mm O.D. glass tube into the $Ba(OH)_2$ reservoir. The reservoir access port is easily sealed during incubation with a serum bottle stopper. Two glass tubes are added for sparging, venting, and medium sampling. The tops of these tubes are connected with a short section of flexible tubing during incubation.

(ii) *Reagents and stock solutions.* (A) Stock solutions, I, II, and III under the following Table 1.

(B) Yeast extract.

(C) Vitamin-free casamino acids.

(D) 70 percent $O_2$ in nitrogen or $CO_2$-free air.

(E) 0.2N $Ba(OH)_2$.

(F) 0.1 N HCl.

(G) 20 percent $H_2SO_4$.

(H) Phenolphthalein.

(I) Dilution water—distilled, deionized water (DIW).

(iii) *Soil Inoculum.* A fresh sample of an organically rich soil is used as the inoculum in the ultimate biodegradation test. Soil is collected, prepared, and stored according to the recommendations of Pramer and Bartha (1972) under paragraph (d)(2) of this section. The soil surface is cleared of litter and a soil sample is obtained 10 to 20 cm below the surface. The sample is screened through a sieve with 2 to 5 mm openings and stored in a polyethylene bag at 2 to 4 °C for not more than 30 days prior to use. The soil is never allowed to air-dry, and shall not be frozen during storage.

TABLE 1—MEDIUM EMPLOYED FOR ASSAY OF $CO_2$ EVOLUTION

| Solution[1] | Compound | Stock Solution Conc. (g/L) |
|---|---|---|
| I | $NH_4Cl$ | 35 |
| | $KNO_3$ | 15 |
| | $K_2HPO_4 \cdot 3H_2O$ | 75 |
| | $NaH_2PO_4 \cdot H_2O$ | 25 |
| II[2] | $KCl$ | 10 |
| | $MgSO_4$ | 20 |
| | $FeSO_4 \cdot 7H_2O$ | 1 |
| III | $CaCl_2$ | 5 |
| | $ZnCl_2$ | 0.05 |
| | $MnCl_2 \cdot 4H_2O$ | 0.5 |
| | $CuCl_2$ | 0.05 |
| | $CoCl_2$ | 0.001 |
| | $H_3BO_3$ | 0.001 |
| | $MoO_3$ | 0.0004 |

[1] = Each liter of test medium contains 1 mL of each solution.

[2] = Final pH is adjusted to 3.0 with 0.10 N HCl.

(iv) *Acclimation Medium.* Acclimation medium is prepared by adding, for each liter of distilled, deionized water (DIW): 1 mL each of solutions I, II, and III in Table 1 in paragraph (b)(1)(iii) of this section, 1.0 gm of soil inoculum (prepared according to paragraph (b)(1)(iii) of this section), 2.0 mL of aerated mixed liquor (obtained from an activated sludge treatment plant not more than 2 days prior to commencing the acclimation phase, and stored in the interim at 4 °C) and 50 mL raw domestic influent sewage. This medium is mixed for 15 minutes and filtered through a glass wool plug in a glass funnel. The filtrate is permitted to stand for 1 hour, refiltered through glass wool, and supplemented with 25 mg/L each of Difco vitamin-free casamino acids and yeast extract. Appropriate volumes are added to 2-liter Erlenmeyer flasks. Test compounds are added incrementally during the acclimation period at concentrations equivalent to 4, 8, and 8 mg/L carbon on days 0, 7, and 11, respectively. On day 14, the medium is refiltered through glass wool prior to use in the test. For evaluating the biodegradability of a series of functionally or structurally related chemicals, media from all inoculum flasks may be combined before final filtration.

(2) *Procedures.* (i) Inoculum (100 mL of acclimation medium) is added to

900 mL DIW containing 1 mL each of solutions I, II, and III in Table 1 under paragraph (b)(1)(iii) of this section in a 2-liter Erlenmeyer flask. Test compound equivalent to 10 mg/liter carbon is added to each of the replicate flasks containing the test medium. Ten mL of 0.2 N Ba $(OH)_2$ are added to the suspended reservoir in each flask and duplicate 10 mL samples of $Ba(OH)_2$ are also saved as titration blanks for analysis with test samples. Flasks are sparged with $CO_2$-free air (for volatile test materials, sparging is done prior to addition of the chemical), sealed, and placed on a gyrotary shaker (approximately 125 rpm) at 20 to 25 °C in the dark. For each set of experiments, each test, reference, inhibited, and control system should be analyzed at time zero and at a minimum of four other times from time zero through day 28. Sampling must be made with sufficient frequency to allow for a smooth plot of biodegradation with time. Sampling times should be varied by the investigator as deemed appropriate to match the rate of degradation of the test substance. Tests may be terminated when biodegradation reaches a plateau and is consistent (±10 percent) over 3 consecutive days or on day 28, whichever occurs first. For chemicals which are water soluble at the test concentration, an adequate volume (5 to 10 mL) of medium is removed for DOC analysis. Each sample for DOC analysis should be filtered through a membrane filter of 0.45 micrometer pore diameter before DOC analysis. For all test and reference compounds, $Ba(OH)_2$ from the center well is removed for analysis. The center well is rinsed with 10 mL $CO_2$-free DIW and is refilled with fresh base. Rinse water is combined with the $Ba(OH)_2$ sample to be analyzed. Flasks are resealed and placed on the shaker. On the day prior to terminating the test, 3 mL of 20 percent $H_2SO_4$ are added to the medium to release carbonate bound $CO_2$.

(ii) For each set of experiments, each test substance shall be tested in triplicate.

(iii) For each set of experiments, one or two reference compounds are included to assess the microbial activity of the test medium. Duplicate reference flasks are prepared by adding reference compound equivalent to 10 mg/liter carbon to each of two flasks containing the test medium. Reference compounds which are positive for ultimate biodegradability include: sodium citrate, dextrose, phthalic acid, trimellitic acid, and aniline.

(iv) For each test set, triplicate controls receiving inoculated medium and no test compound, plus all test and reference flasks, are analyzed for $CO_2$ evolution and DOC removal. Results from analysis of the control flasks (DOC, $CO_2$ evolution, etc.) are subtracted from corresponding experimental flasks containing test compound in order to arrive at the net effect due to the test compound.

(v) A test system containing a growth inhibitor should be established as a control for each substance tested for biodegradation by this method. That inhibited system must contain the same amount of water, mineral nutrients, inoculum, and test substance used in the uninhibited test systems, plus 50 mg/L mercuric chloride $(HgCl_2)$ to inhibit microbial activity.

(vi) Flasks shall be incubated in the dark to minimize both photochemical reactions and algal growth. Appropriate sterile controls or controls containing a metabolic inhibitor, such as 50 mg/1 $HgCl_2$, are needed to correct for interferences due to nonbiological degradation. With volatile organic materials, sparging with $CO_2$-free air is performed only once, just prior to addition of the test chemical. Analyses for $CO_2$ evolution and DOC removal are conducted within 2 to 3 hours of sampling to minimize interferences which may occur in storage. All glassware should be free of organic carbon contaminants.

(3) *Analytical measurements.* The quantity of $CO_2$ evolved is measured by titration of the entire $Ba(OH)_2$ sample (10 mL $Ba(OH)_2$ + 10 mL rinse water) with 0.1 N HCl to the phenolphthalein end point. $Ba(OH)_2$ blanks are also supplemented with 10 mL $CO_2$-free DIW and titrated in a similar manner. Samples (5 mL) for DOC are centrifuged and/or filtered and supernatant or filtrate analyzed by a suitable total organic carbon method.

(e) *Data and reporting*—(1) *Treatment of results.* (i) Test compound (10 mg carbon) is theoretically converted to 0.833 mmol $CO_2$. Absorbed $CO_2$ precipitates as $BaCO_3$ from $Ba(OH)_2$, causing a reduction in alkalinity by the equivalent of 16.67 mL of 0.1 N HCl for complete conversion of the test compound carbon to $CO_2$. Therefore, the percent theoretical $CO_2$ evolved from the test compound is calculated at any sampling time from the formula:

Percent $CO_2$ evolution = [(TF-CF)/16.67] 100 (for 10 mg/L test compound carbon)

where:

TF=mL 0.1 N HCl required to titrate $Ba(OH)_2$ samples from the test flask
CF=mL 0.1 N HCl required to titrate $Ba(OH)_2$ samples from the control flask.

(ii) The cumulative percent $CO_2$ evolution at any sample time is calculated as the summation of the percent $CO_2$ evolved at all sample points of the test.

(iii) The percent DOC disappearance from the test compound is calculated from the following equation:

Percent DOC Removal = $[1-(DTF_x-DCF_x)/(DTF_o-DCF_o)]$ 100

where:

DTF=Dissolved organic carbon from test flask
DCF=Dissolved organic carbon from control flask
o=Day zero measurements
x=Day of measurements during test.

(iv) The difference between the amount of 0.1 N HCl used for the $Ba(OH)_2$ titration blank samples and the $Ba(OH)_2$ samples from the control units (no test compound) is an indication of the activity of the microorganisms in the test system. In general, this difference is approximately 1 to 3 mL of 0.1 N HCl at each sampling ime. A finding of no difference in the itration volumes between these two amples indicates a poor inoculum. In his case, the validity of the test reults is questionable and the test set hall be rerun beginning with the aclimation phase.

(v) $CO_2$ evolution in the reference lasks is also indicative of the activity f the microbial test system. The suggested reference compounds should all ield final $CO_2$ evolution values of at least 60 percent of theoretical $CO_2$. If, for any test set, the percent theoretical $CO_2$ evolution value for the reference flasks is outside this range, the test results are considered invalid and the test is rerun.

(vi) Inhibition by the test compound is indicated by lower $CO_2$ evolution in the test flasks than in the control flasks. If inhibition is noted, the study for this compound is rerun beginning with the acclimation phase. During the test phase for inhibitory compounds, the test chemical is added incrementally according to the schedule: Day 0 – 0.5 mg/liter as organic carbon, Day 2—1 mg/liter C, Day 4—1.5 mg/liter C, Day 7—2 mg/liter C, Day 10—5 mg/liter C. For this case, the $Ba(OH)_2$ is sampled on Day 10, and weekly thereafter. The total test duration remains 28 days.

(vii) The use of $^{14}C$-labeled chemicals is not required. If appropriately labeled test substance is readily available and if the investigator chooses to use this procedure with labeled test substance, this is an acceptable alternative. If this option is chosen, the investigator may use lower test substance concentrations if those concentrations are more representative of environmental levels.

(2) *Test report.* (i) For each test and reference compound, the following data shall be reported.

(ii) Information on the inoculum, including source, collection date, handling, storage and adaptation possibilities (i.e., that the inoculum might have been exposed to the test substance either before or after collection and prior to use in the test).

(iii) Results from each test, reference, inhibited (with $HgCl_2$) and control system at each sampling time, including an average result for the triplicate test substance systems and the standard deviation for that average.

(iv) Average cumulative percent theoretical $CO_2$ evolution over the test duration.

(v) Dissolved organic carbon due to test compound at each sampling time (DTF-DCF).

(vi) Average percent DOC removal at each sampling time.

(vii) Twenty-eight day standard deviation for percent $CO_2$ evolution and DOC removal.

(d) *References*. For additional background information on this test guideline the following references should be consulted:

(1) Gledhill, W.E. "Screening Test for Assessment of Ultimate Biodegradability: Linear Alkyl Benzene Sulfonate," *Applied Microbiology*, 30:922-929 (1975).

(2) Pramer, D., Bartha, R. "Preparation and Processing of Soil Samples for Biodegradation Testing," *Environmental Letters*, 2:217-224 (1972).

[50 FR 39252, Sept. 27, 1985, as amended at 52 FR 19058, May 20, 1987]

## § 796.3140 Anaerobic biodegradability of organic chemicals.

(a) *Introduction*—(1) *Purpose*. (i) This section has been developed for screening for anaerobic biodegradability of organic chemicals. A high biodegradability result in this test provides evidence that the test substance will be biodegradable in sewage-treatment plant anaerobic digestors and in many natural anaerobic environments such as swamps, flooded soils and surface water sediments.

(ii) On the contrary, a low biodegradation result may have other causes than poor biodegradability of the test substance. Inhibition of the microbial inoculum by the substance at the test concentration may be observed. In such cases further work is needed to assess the anaerobic biodegradability and to determine the concentrations at which toxic effects are evident. An estimate of the expected environmental concentration will help to put toxic effects into perspective.

(2) *Principle of the test method*. (i) This section is based on anaerobic biodegradability methods referenced in paragraph (d) of this section.

(ii) A chemically defined anaerobic medium, containing resazurin as an oxidation/reduction indicator and 10 percent (v/v) primary anaerobic digestor sludge from a waste treatment plant, is dispensed in 100 mL portions into 160 mL capacity serum bottles. Selected bottles are supplemented with test substance at a concentration equivalent to 50 mg/L as organic carbon. Gas production is measured with a pressure transducer. The extent of biodegradation is determined by comparing gas production from blank control bottles and bottles containing the test substance.

(iii) The average cumulative gas production ($CH_4$ + $CO_2$), in mL, is reported for blank controls, solvent controls, test substances and any reference compounds. Also reported is the percent of theoretical anaerobic biodegradation at test completion or 56 days (whichever comes first) and the standard deviation between replicate bottles.

(3) *Prerequisites*. The total organic carbon content of the test material shall be calculated or, if this is not possible, analyzed, to enable the theoretical yield of carbon dioxide and methane to be calculated.

(4) *Guidance information*. (i) Information on the relative proportions of the major components of the test material will be useful in interpreting the results obtained, particularly in those cases where the result lies close to a "pass level."

(ii) Information on the toxicity of the chemical may be useful in the interpretation of low results and in the selection of appropriate test concentrations.

(5) *Reference substances*. In some cases, when investigating a substance, reference substances may be useful and an inventory of suitable reference substances needs to be identified. In order to check the activity of the inoculum, the use of a reference substance is desirable. Ethanol may be used for this purpose. The ethanol must exhibit anaerobic biodegradation (as gas production) greater than 50 percent of the theoretical maximum within 56 days. Otherwise the test is regarded as invalid and shall be repeated using an inoculum from a different source.

(6) *Applicability*. The method is only applicable to those organic test substances which, at the concentration used in the test, are not inhibitory to bacteria.

(7) *Reproducibility*. The reproducibility of the method has not yet been determined; however, it is believed to be appropriate for a screening test

which has solely an acceptance but no rejective function.

(8) *Sensitivity.* The sensitivity of the method is largely determined by the necessity to compare gas production in test-substance bottles with gas production in blank control bottles. The method suggests the use of test substance at a concentration of 50 mg/L as organic carbon. This concentration will produce a maximum of 10.5 mL of $CH_4$ and $CO_2$ gases at 35 °C. Actual measured gas production will be less due to (i) incomplete conversion of all of the organic carbon into $CH_4$ and $CO_2$ and (ii) the extent to which the $CO_2$ and $CH_4$ remain solubilized in the aqueous phase. The use of test substance at 50 mg/L as organic carbon represents a compromise between the desire to maximize gas production and thus the sensitivity of the test, and the desire to minimize the possibility of toxicity to the microbial population.

(9) *Possibility of standardization.* This possibility exists. The major difficulty is to standardize the inoculum in such a way that interlaboratory reproducibility is ensured.

(10) *Possibility of automation.* None at present.

(b) *Test procedures*—(1) *Preparations*—(i) *Apparatus.* (A) If gas production is measured with a pressure transducer, apparatus such as a 20-gauge syringe needle attached by means of an inert capillary tube to a three-way valve (Hamilton Mininert valve 3-FLM-IX or equivalent) fitted to a pressure transducer (Unimeasure 100-500 ohm/2mA pressure transducer or equivalent) and an appropriate ohmmeter (e.g., Digitec Model 2120).

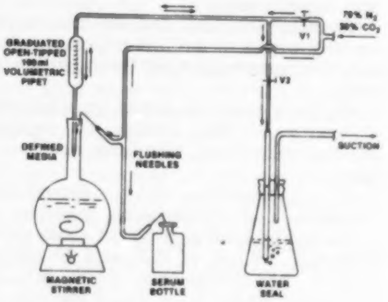
(B) If gas production is measured with a syringe, apparatus such as a 20 mL capacity gas-tight glass syringe fitted with a 20-gauge syringe needle.

(C) If methane ($CH_4$) and carbon dioxide ($CO_2$) are quantified using an analytical procedure specific for $CH_4$ and $CO_2$, apparatus necessary to carry out that analysis, such as a gas chromatograph equipped with a suitable detector.

(D) An incubator sufficient to store the test bottles at 35±1 °C for the duration of the test.

(E) Apparatus suitable for the maintenance of anaerobic conditions during medium preparation and inoculation, such as that shown in the following Figure 1:

FIGURE 1—SCHEMATIC DIAGRAM OF APPARATUS SUITABLE FOR MAINTENANCE OF ANAEROBIC CONDITIONS DURING MEDIUM PREPARATION AND INOCULATION



(F) A supply of 160 mL capacity serum bottles with butyl rubber stoppers.

(ii) *Nutrient medium*—(A) *Stock solutions.* (*1*) S-1 Prepare a solution in distilled water containing resazurin at 0.5 g/L.

(*2*) S-2 Dissolve 20 g ammonium monohydrogen phosphate, $(NH_4)_2HPO_4$, and 100 g ammonium chloride, $NH_4Cl$, in distilled water and dilute to 1 L.

(*3*) S-3 Dissolve 18 g calcium chloride, $CaCl_2$; 180 g magnesium chloride, $MgCl_2.6H_2O$; 130 g potassium chloride, KCl; 2 g manganous chloride, $MnCl_2.4H_2O$; 3 g cobalt chloride, $CoCl_2.4H_2O$; 0.6 g boric acid, $H_3BO_3$; 0.23 g cupric chloride, $CuCl_2$; 1.0 g sodium molybdate, $Na_2MoO_4.2H_2O$ and 0.2 g zinc chloride, $ZnCl_2$, in distilled water and dilute to 1 L.

(*4*) S-4 Dissolve 368 g ferrous chloride, $FeCl_2.4H_2O$, in distilled water and dilute to 1 L.

(*5*) S-5 Dissolve 50 g sodium sulfide, $Na_2S.9H_2O$, in distilled water and dilute to 1L.

(B) *Reagents.* Sodium bicarbonate, $NaHCO_3$.

(iii) *Inoculum.* (A) The inoculum should consist of sludge from an anaerobic sludge digestor. It is recommended that well-mixed primary sludge from a digestor with a sludge retention time of 15 to 25 days be used. At the time of collection, the sludge should be sieved through a 2 mm mesh screen.

(B) Most sludges can be stored for up to 2 weeks at 4 °C, if necessary, but it is recommended that fresh sludge be used.

(C) Care shall be taken to minimize exposure of the sludge to oxygen during collection, handling and storage.

(2) *Procedure*—(i) *Inoculated medium.* (A) Prereduced medium is prepared by adding 8 mL of stock solution S-1, 8 mL of S-2, and 40 mL of S-3 to approximately 3,500 mL of deionized water in a 4-L Florence or Erlenmeyer flask. This medium is heated to a boil, while being stirred with a magnetic stir bar and sparged with oxygen ($O_2$)-free nitrogen. The $O_2$-free nitrogen is obtained by passing nitrogen gas through a quartz cylinder filled with copper filings heated to 400 °C. Alternatively, commercial nitrogen free of oxygen may be used.

(B) The flask containing the medium is placed in an ice bath and $O_2$-free carbon dioxide ($CO_2$) is introduced into the stream of $O_2$-free nitrogen to a concentration in the gas stream of 30 percent (v/v).

(C) When the medium has cooled to 35 °C, the flask is removed from the ice bath and the following components are added: 4 mL of solution S-4; 20 mL of solution S-5; 10.56 g sodium bicarbonate; and 400 mL of sludge inoculum. The final volume should be approximately 4 L.

(ii) *Filling test bottles.* (A) One-hundred mL portions of the inoculated medium are transferred anaerobically into serum bottles with a total capacity of about 160 mL. An apparatus suitable for the maintenance of anaerobic conditions during medium preparation and transfer is shown in Figure 1. V1 and V2 are valves that are used to control the transfer of medium to the serum bottles. Inoculated medium is drawn into the pipet by suction, the pipet is moved and the tip inserted into a serum bottle. During these processes the serum bottle and neck of the medium flask are continually sparged with the oxygen-free mixture of $N_2$ and $CO_2$.

(B) The medium in the pipet is discharged into the serum bottle.

(C) A new butyl rubber serum-bottle stopper is inserted into the neck of the bottle while the needle being used to sparge the contents with the $N_2$ and $CO_2$ mixture is removed.

(iii) *Test and reference chemicals.* (A) Test and reference chemicals are added to serum bottles to yield a final concentration of 50 mg/L as organic carbon. These chemicals may be added to the bottles prior to the addition of inoculated medium or following medium addition, depending upon the nature of the test or reference substance and whether or not it must be added to the bottles dissolved in a volatile solvent.

(B) Test or reference chemicals with sufficiently high water solubility may be added to test bottles from a neutralized stock solution. The stock solution shall be prepared so that a minimal volume is needed to yield 50 mg/L as organic carbon in the medium.

(C) Suitable liquid test or reference chemicals may be added directly by injection from a calibrated syringe.

(D) Test or reference chemicals with relatively low water solubility may be added to test bottles by a direct addition of weighed amounts or by using an organic solvent. Direct addition is recommended. If a volatile organic solvent is used, a suitable procedure is to dissolve the chemical in the solvent, pipet an appropriate amount into the bottle, allow the solvent to evaporate, and then add the inoculated medium. Diethyl ether is a suitable solvent for many organics, but it must be completely removed from the bottle because it will adversely affect methanogenesis. If an organic solvent is used without removal of the solvent before the test, the solvent must neither significantly inhibit nor contribute to apparent gas production. Acetonitrile, dioxane and pyridine have been found acceptable for this purpose.

(E) Bottles containing inoculated medium but no test or reference chemical are employed in each test. These are the blank controls.

(F) If an organic solvent is used to add chemical to test bottles without evaporation of the solvent before the test, bottles containing inoculated medium and an equivalent amount of the organic solvent, but no test or reference chemical, must be employed for each solvent used. These are the solvent controls.

(G) The substance bottles, blank controls, and solvent controls, shall be prepared in triplicate.

(iv) *Incubation.* (A) At the start of the incubation, pressure in each bottle must be released.

(B) Bottles are incubated in the dark at 35±1 °C for 8 weeks or until biodegradation is complete. Bottles containing oxidized (pink) resazurin should be discarded.

(3) *Analytical measurements.* (i) A sufficient number of measurements of gas pressure or gas volume shall be made to establish the pattern of gas production with time. Measurements are generally made weekly for up to 8 weeks. The frequency of measurements may be varied by the investigator as deemed appropriate to match the degradation rate of the chemical.

(ii) Gas production is measured for each bottle, using a pressure transducer, syringe or other suitable apparatus.

(iii) The use of a pressure transducer is recommended. The ohmmeter should be calibrated by injecting known volumes of gas into bottles containing medium and a standard curve of gas volume vs. meter reading plotted. Excess pressure should be vented after each measurement so that all bottles will have the same internal pressure following each measurement time.

(iv) If a syringe is used to measure gas volume, the following procedure is recommended. The syringe is flushed with 30 percent (v/v) $CO_2$ in $O_2$-free nitrogen. The syringe is held in a horizontal position during the measurement, taking care to keep the needle within the gas space of the serum bottle. Gas production is determined by allowing the syringe plunger to move freely to equalize the vessel and atmospheric pressures.

(v) Methane and carbon dioxide may be determined using analytical methods suitable for the detection and quantification of those compounds.

(c) *Data and reporting*—(1) *Treatment of results.* (i) Cumulative average gas volume from the anaerobic biodegradation of test or reference substances is calculated by subtracting the cumulative average gas volume production for triplicate blank controls (or solvent controls, if an organic solvent was included) from the average value for triplicate test or reference substance bottles at the same incubation time. The percent of theoretical gas volume produced is calculated by dividing cumulative average gas volume from test or reference chemical by the theoretical maximum gas production and multiplying by 100.

(ii) The maximum methane plus carbon dioxide production theoretically obtainable from an organic chemical in this test is 10.5 mL, if the starting concentration is 50 mg/L as organic carbon. This can be calculated as shown below, using benzoic acid as an example.

$C_7H_6O_2 + 12\ H_2O \rightarrow 7\ CO_2 + 15\ H_2$

$7\ CO_2 + 15\ H_2 \rightarrow 3.75\ CH_4 + 3.25\ CO_2 + 7.5\ H_2O$

---

$C_7H_6O_2 + 4.5\ H_2O \rightarrow 3.75\ CH_4 + 3.25\ CO_2$

(iii) At a concentration of 50 mg/L as organic carbon in a 100 mL aqueous phase at 35 °C, the maximum volume of gas produced is calculated as follows:

At 50 mg/L as carbon, in 100 mL there are 7.27 mg benzoic acid.

7.27 mg benzoic acid = 0.0595 m moles.
$0.0595 \times 3.75 = 0.2232$ m moles $CH_4$
$0.0595 \times 3.25 = 0.1934$ m moles $CO_2$
——— = 0.4167 m moles total gas production.

At 35° and atmospheric pressure, one mole of gas occupies approximately 25.25 liters.

Thus, 0.4167 m moles at 35° will occupy 10.5 mL.

(iv) Likewise, any test compound added at a concentration that provides 5 mg of organic carbon to the test bottle will have a theoretical maximum gas production of 10.5 mL.

(2) *Test report*. The following shall be reported: (i) Information on the inoculum including information on the source, retention time, percent volatile solids, date of collection, storage, handling and adaption possibilities (i.e., information on the possibility that the inoculum was exposed to the test chemical or related chemicals before the test). Retention time and percent volatile solids of the sludge are generally obtainable from the waste treatment plant operator.

(ii) Average cumulative gas production (mL) from blank control bottles, solvent control bottles, test substance bottles and reference compound bottles at each measurement time.

(iii) Percent of theoretical anaerobic biodegradation for each test substance and reference compound at each measurement time.

(iv) The standard deviation for each replicate set of bottles at the final measurement time.

(v) A plot of the percent of theoretical anaerobic biodegradation vs. time for each test substance and reference compound.

(vi) A description of any deviations from this test guideline, such as variations in the medium or the concentration of test substance, test conditions, and analytical techniques.

(d) *References*. For additional background information on this test guideline, the following references should be consulted:

(1) Gossett, J.M., Healy, J.B., Stuckey, D.C., Young, L.Y., and McCarty, P.L. "Heat treatment of refuse for increasing anaerobic biodegradability," *Stanford University Civil Engineering Technical Report No. 205*, (1976).

(2) Healy, J.B., Owen, W.F., Stuckey, D.C., Young, L.Y., and McCarty, P.L. "Heat treatment of organics for increasing anaerobic biodegradability," *Stanford University Civil Engineering Technical Report No. 222*, (1977).

(3) Healy, J.B. and Young, L.Y. "Degradation of simple aromatic compounds under methanogenic conditions," *Abstracts—Annual Meeting American Society for Microbiology*, Vol. 013 (1977), p. 263.

(4) U.S. Environmental Protection Agency. Healy, J.B. and Young, L.Y. "Methanogenic biodegradation of aromatic compounds," Workshop: Microbial Degradation of Pollutants in Marine Environments, EPA Report No. 600/9-79-012 (Gulf Breeze, Florida, 1978).

(5) Owen, W.F., Stuckey, D.C., Healy, J.B., Jr., Young, L.Y. and McCarty, P.L. "Bioassay for Monitoring Biochemical Methane Potential and Anaerobic Toxicity," *Water Research*, 13:485-492 (1979).

(6) Healy, J.B., Jr., and Young, L.Y. "Anaerobic Biodegradation of Eleven Aromatic Compounds to Methane," *Applied Environmental Microbiology*, 38:84-89 (1979).

(7) Miller, T.C., and Wolin, M.J. "A Serum Bottle Modification of the Hungate Technique for Cultivating Obligate Anaerobes," *Applied Microbiology*, 27:985-987 (1974).

[50 FR 39252, Sept. 27, 1985, as amended at 52 FR 19058, May 20, 1987; 53 FR 49140, Dec. 6, 1988; 54 FR 21064, May 16, 1989]

### § 796.3180 Ready biodegradability: Modified AFNOR test.

(a) *Introductory information*—(1) *Prerequisites*. (i) The test material must be at least soluble at the concentration to be tested, 40 mg dissolved organic carbon/liter (DOC/l).

(ii) The organic carbon content of the test material must be established.

(2) *Guidance information*. (i) Information on the relative proportions of the major components of the test material will be useful in interpreting the results obtained, particularly in those cases where the result lies close to the "pass level."

(ii) Information on the toxicity of the chemical may be useful in the interpretation of low results and in the selection of appropriate test concentrations.

(3) *Interpretation of results*. Because of the stringency of this test a result of less than 70 percent loss of DOC (within 28 days) does not necessarily mean that the test compound is not biodegradable under environmental conditions, but indicates that more work will be necessary to establish biodegradability.

(4) *Qualifying statements.* (i) The method is only applicable to those organic test materials which, at the concentration used in the test:

(A) Are soluble in water.

(B) Have negligible vapour pressure.

(C) Are not inhibitory to bacteria.

(D) Do not significantly adsorb on glass surfaces.

(ii) This test has been found suitable by the OECD Expert Group Degradation/Accumulation for determining the ready biodegradability of organic chemicals under aerobic conditions. It has been tested in the OECD Laboratory Intercomparison Test Program (1978-1980).

(5) *Recommendations.* (i) Test Chemicals giving a result of greater than 70 per cent loss of DOC (within 28 days) should be regarded as readily biodegradable. This level must be reached within 10 days of biodegradation exceeding 10 percent.

(ii) If the limits of sensitivity of organic carbon analyzers are improved, the use of lower test concentrations may be an advantage, particularly for toxic compounds.

(6) *Standard documents.* This test is based on "Norme Experimentale AFNOR T 90-302."

(b) *Method*—(1) *Introduction, purpose, scope, relevance, application and imits of test.* (i)(A) The method decribed in this test guideline allows the valuation in aqueous medium of soalled "total" biodegradability under xperimental conditions which are asy to establish. These conventional onditions do not necessarily correpond in all cases to the optimal condiions which would result in the maxial value of biodegradability.

(B) The method is applicable to hose organic products, alone or in ixtures, which are:

(*1*) Soluble in water at the concenration used under the conditions of he test.

(*2*) Non-volatile or at least have a egligible vapour pressure under the onditions of the test.

(*3*) At the concentration foreseen in he test, not inhibitory with respect to he bacteria responsible for the bioderadation.

(ii) *Reference substances.* (A) In ome cases when investigating a new substance reference substances may be useful; however, specific reference substances cannot yet be recommended. Aniline, sodium acetate or sodium benzoate may be used for this purpose; they must exhibit DOC removal of >70 percent within 28 days, otherwise the test is regarded as invalid and should be repeated using an inoculum from a different source.

(B) In this specific test glucose is used especially for the inhibition test and may be used also to check the activity of the inoculum.

(iii) *Principle of the test method.* The biodegradation of organic products dissolved in water by chemico-organotrophic micro-organisms using the products as the sole source of carbon and energy is observed. These products are studied at a concentration such that the initial content of organic carbon is 40 mg/l. The organic carbon remaining in solution after 3, 7, 14, 28 (and 42) days is measured and the corresponding level of biodegradation calculated. The biodegradability is evaluated on the basis of this level.

(iv) *Quality criteria.* This is an evaluation method and not a method for the determination of biodegradability.

(A) *Reproducibility.* To date no OECD ring test has been performed especially for reproducibility, but some general ring tests have been made with the CEFIC and with AFNOR, which have some relevance.

(B) *Sensitivity.* This method is sensitive to an order of magnitude of ±10 percent.

(C) *Specificity.* This Test Guideline is applicable to organic products soluble in the test medium at a concentration equal at least to one containing 40 mg/l as carbon.

(D) *Possibility of standardization.* This method may be standardized in the course of work by ISO.

(E) *Possibility of automation.* Automation seems possible.

(2) *Description of the test procedure*—(i) *Preparations*—(A) *Site.* The incubation must be carried out in the absence of any intense lighting, in an incubator maintained at 25±1 °C and free from toxic vapors.

(B) *Reagents.* (*1*) The chemical products used must be of recognized analytical purity.

(*2*) Distilled water must not contain more than 2 mg/l of organic carbon. In any case the organic carbon content of the distilled water must not exceed 10 percent of the organic carbon level introduced by the test substance.

(*3*) Test medium: (*i*) Prepare the test medium as indicated, using sterile material. For one litre of solution, dissolve the following in distilled water:

| | |
|---|---|
| Ammonium sulphate $(NH_4)_2SO_4$ | 0.300 g. |
| Ammonium nitrate $NH_4NO_3$ | 0.150 g. |
| Monopotassium phosphate $KH_2PO_4$. | 0.300 g. |
| Disodium phosphate $Na_2HPO_4 \cdot 12H_2O$. | 2.000 g. |
| Magnesium sulphate $MgSO_4 \cdot 7H_2O$. | 0.050 g. |
| Calcium chloride $CaCl_2 \cdot 2H_2O$ | 0.050 g. |
| Yeast extract | 0.005 g. |

The pH is 7.5±0.1.

(*ii*) Add 1 ml of trace element solution of the following composition:

| | |
|---|---|
| Ferrous sulphate $FeSO_4 \cdot 7H_2O$ | 0.100 g. |
| Manganese sulphate $MnSO_4 \cdot H_2O$. | 0.100 g. |
| Potassium molybdate $K_2MoO_4$ | 0.025 g. |
| Sodium tetraborate $Na_2B_4O_7 \cdot 10H_2O$. | 0.025 g. |
| Cobalt nitrate $Co(NO_3)_2 \cdot 6H_2O$ | 0.025 g. |
| Copper chloride $CuCl_2 \cdot 2H_2O$ | 0.025 g. |
| Zinc chloride $ZnCl_2$ | 0.025 g. |
| Ammonium metavanadate $NH_4VO_3$. | 0.010 g. |
| Distilled water to | 100 ml. |

(*4*) The trace element solution can be kept for one month at a temperature between +1 and +4 °C.

(*5*) Make up to the volume stated (1 liter) and mix. The medium must be used within 12 hours.

(C) *Apparatus*. Usual laboratory items, and

(*1*) Apparatus for the assay of organic carbon.

(*2*) Spectrophotometer.

(*3*) Centrifuge, 4000 $ms^{-2}$

(*4*) Shaker allowing adequate aeration and shaking.

(*5*) Apparatus for the assay of dissolved oxygen.

(*6*) pH meter.

(*7*) 500-ml wide-neck conical flasks, sterile.

(*8*) Apparatus for sterile filtration. The glassware must be thoroughly cleaned and in particular free from all traces of organic or toxic matter.

(ii) *Procedure*—(A) *Preparation of the test solutions.* (*1*) Prepare the following solutions:

(*i*) Solution of tested product in the test medium in such a way as to obtain a concentration of 40 mg/l of organic carbon.

(*ii*) Solution of glucose in the test medium in such a way as to obtain a concentration of 40 mg/l of organic carbon.

(*iii*) Solution containing in the test medium the concentrations of product and glucose used for the preparation of solutions.

(*iv*) An adequate volume of test medium should be available.

(*2*) Mix the four solutions individually and sterilize by filtration through a membrane filter of 0.22 μm porosity.

(B) *Preparation of the inoculum.* (*1*) Take an adequate volume of a mixture of three samples from polluted surface water (effluent from town sewage works) free from major specific pollutants. The bacterial count for each sample must be at least $10^5$ bacteria/ml.

(*2*) The samples must be used for the inoculation within a period of 12 hours, including transportation, and must not remain for more than 6 hours without aeration.

(*3*) Filter through paper to eliminate the larger insoluble particles, collect the filtrate and pass through a membrane filter of pore size 0.22 μm. Wash with any isotonic solution. Take up the bacteria deposited on the membrane filter in a small volume of solution or with any other isotonic solution. Mix well. Measure the absorbance at 620 nm and from this deduce the concentration of bacteria in relation to a standard curve obtained previously by means of solid medium counts of *Pseudomonas fluorescens* strain ATCC 15453. Add the volume of solution required to adjust the concentration of bacteria to $5 \pm 3 \times 10^7$/ml. Use the inoculum within the next hour.

(C) *Actual test performance.* (*1*) All the necessary manipulations must be carried out by sterile methods. Divide solutions into the test flasks (previously sterilized) according to the following scheme:

| | |
|---|---|
| Flask No. 1 (test) | 150 ml solution 1. |
| Flask No. 2 (test) | 150 ml solution 1. |
| Flask No. 3 (test) | 150 ml solution 1. |
| Flask No. 4 (sterile control). | 150 ml solution 1. |
| Flask No. 5 (glucose control). | 150 ml solution 2. |
| Flask No. 6 (control of inhibitory action). | 150 ml solution 3. |
| Flask No. 7 (inoculum control). | 150 ml solution 4. |

(*2*) Seed flasks 1, 2, 3, 5, 6 and 7 with 1.5 ml of inoculum and mix well by manual shaking.

(*3*) Take an aliquot of 3-5 ml from each flask. Centrifuge the aliquots at 4000 ms$^{-2}$ for 15 minutes, keeping the temperature below 26 °C. In certain cases, and especially in presence of relatively low specific gravity insoluble fractions, it may be necessary to centrifuge for a longer time or with a higher acceleration. Collect the supernatants for assays of organic carbon at time 0.

(*4*) Place the flasks on the shaker and leave them there throughout the test period; the shaking must be such that the validity clause is fulfilled.

(*5*) In the same way as for the assay of organic carbon at time 0, carry out this assay on flasks 1, 2, 3, 5, 6 and 7 after 3, 7, 14, 28 (and 42) days of incubation. However, if the reduction in carbon content reaches 95 percent of the initial content in flasks 1, 2 and 3, consider the test as ended.

(*6*) The test can be finished before the 28th day in the case where a plateau is observed before the 28th day. In the case where a degradation has obviously started on day 28 but has not reached a plateau on day 28, it is considered good practice to extend the experiment for 1 of 2 weeks longer.

(*7*) At the end of the test carry out an assay of organic carbon in flask 4 in the same manner as at time 0.

(*8*) If the assays of organic carbon contents have to be deferred, keep the supernatant at 4 °C in the dark in hermetically sealed glass flasks; the maximum acceptable duration of preservation is 24 hours. If the analysis cannot be carried out within 24 hours, then freeze at a temperature below −18 °C.

(*9*) To compensate for loss of water due to evaporation, before each sampling verify the volume of medium in the flask and, if necessary, fill up with distilled water sterilized by filtration through a membrane of 0.22 μm pore size to restore the volume measured after the previous sampling.

(c) *Data and reporting*—(1) *Treatment of the results.* (i) Calculation:

(A) Determine the percentage elimination of soluble organic carbon for each sampling, using the following formula:

$$\%\ \text{elimination of carbon at time } t = \frac{(C_o - C_{oi})(C_t - C_{ti})}{(C_o - C_{oi})} \times 100$$

here

=the mean level of organic carbon at time 0 in flasks 1, 2 and 3 (or the organic carbon content at time 0 in one of the flasks 4, 5 or 6).

=the organic carbon level in flask 7 at time 0.

=the mean level of organic carbon at time t in flasks 1, 2 and 3 (or the organic carbon level at time t in one of the flasks 4, 5 or 6).

=the organic carbon level in flask 7 at time t.

(B) The level of biodegradation is the percentage elimination of the organic carbon.

(C) Set up a table of biodegradation levels as a function of time. Draw the curve of the change in biodegradation level as a function of time when a large enough number of significant points is available.

(ii) Note: The comparison of the percentage eliminations of carbon in flasks 1, 2 and 3, on the one hand, and in flask 4, on the other hand, allows the causes of the degradation observed to be differentiated: the physical-chemical mechanisms in flask 4 and the physical-chemical plus biological mechanisms in flasks 1, 2 and 3.

(2) *Validity of the results.* If one of the following conditions (i) to (iii) is not fulfilled, begin the test anew. In the case of condition (i), the level of shaking must be increased in the new test.

(i) The concentration of dissolved oxygen on day 3 in flask 5 must be at least 5 mg/l.

(ii) The level of glucose degradation in flask 5 must be at least 80 percent by day 7.

(iii) At the end of the test, flask 4 must still be sterile. Check this by seeding into a tube of liquid culture medium (Dehydrated yeast extract, 3 g; Pancreatic caseine peptone, 6 g; Water, 1000 ml—Dissolve the components or the dehydrated complete medium in boiling water. If necessary, adjust the pH in such a way that after sterilisation it is 7.2±0.2 at 20 °C) and incubating at 25 °C for 5 days. The level of glucose biodegradation in flask 6 must, by day 7, be at least 75 percent of that observed in flask 5. If this limit is not reached, consider that the product subjected to the test presents an inhibitory effect towards the bacteria present and that the method is not applicable to it at the concentration laid down by this standard.

(3) *Interpretation of results.* See paragraph (a) of this section.

(4) *Test report.* (i) Mention the following points in particular:

(A) Motivation for any rejection of the test.

(B) Extent of the disappearance of the product in flask 4 at the end of the test.

(C) Any inhibition phenomena observed.

(ii) *Biodegradability:* Express the biodegradability as the highest level of biodegradation noted during the period of 28 days. The course of the degradation should be displayed graphically in a diagram.

[50 FR 39483, Sept. 27, 1985]

## § 796.3200 Ready biodegradability: Closed bottle test.

(a) *Introductory information*—(1) *Prerequisites.* The empirical formula of the test material is required so that the theoretical oxygen demand (TOD) may be calculated. If this is unknown, the chemical oxygen demand (COD) of the test material may serve as the reference point (6).

(2) *Guidance information.* (i) Information on the relative proportions of the major components of the test material will be useful in interpreting the results obtained, particularly in those cases where the result lies close to the "pass level."

(ii) Information on the toxicity of the chemical may be useful in the interpretation of low results and in the selection of appropriate test concentrations.

(3) *Interpretation of results.* Because of the stringency of this test, a measured BOD (biochemical oxygen demand) which is less than 60 percent of TOD (within 28 days) does not necessarily mean that the test compound is not biodegradable under environmental conditions, but indicates that more work will be necessary to establish biodegradability. The possibility that nitrogen-containing compounds may affect the results should be considered.

(4) *Qualifying statements.* (i) The method is only applicable to those organic test materials which, at the concentration used in the test, are not inhibitory to bacteria.

(ii) This test has been found suitable by the OECD Expert Group Degradation/Accumulation for determining the ready biodegradability of organic chemicals under aerobic conditions. It has been tested in the OECD Laboratory Intercomparison Test Program (1978–1980).

(5) *Recommendations.* (i) Test chemicals giving a measured BOD which is greater than 60 percent of the TOD (within 28 days) should be regarded as readily biodegradable. This level must be reached within 10 days of biodegradation exceeding 10 percent.

(ii) If the test material is not soluble at the test concentration, special measures, such as the use of ultrasound dispersion, may have to be employed to achieve a good dispersion of test material.

(6) *Standard documents.* This test guideline is based on a paper found under paragraph (d)(8) of this section.

(b) *Method*—(1) *Introduction, purpose, scope, relevance, application and limits of test.* (i) The purpose of the method is the measurement of the biodegradability of organic compounds in an aerobic, aqueous medium at a test concentration of 2 (standard concentration) to 10 mg/l of active material. Most data elaborated with this test pertain to water-soluble compounds; however, volatile compounds and those of low water solubility may also be tested, at least in principle.

(ii) *Definitions and units.* The degradation is stated as the biochemical oxygen demand (BOD) within 28 days as a percentage of either the theoretical oxygen demand (TOD) or the chemical oxygen demand (COD).

For example:

$$\text{Percent biodegradability} = \frac{\text{mg } O_2/\text{mg active substance}}{\text{TOD}} \times 100$$

or

$$\text{Percent biodegradability} = \frac{\text{mg } O_2/\text{mg active substance}}{\text{COD}} \times 100$$

where

TOD=theoretical oxygen demand (calculation, see Data and reporting, below)

COD=chemical oxygen demand determined experimentally.

(iii) *Reference substances.* In some cases when investigating a new substance, reference substances may be useful; however, specific reference substances cannot yet be recommended. In order to check the activity of the inoculum, the use of control substances is desirable. Aniline, sodium acetate, sodium benzoate, sodium n-dodecylsulfate and the sodium salt of linear alkylbenzenesulfonic acid (8, 9) may be used for this purpose. They must exhibit a BOD which is >60 percent of their TOD within 28 days, otherwise the test is regarded as invalid and should be repeated using an inoculum from a different source.

(iv) *Principle of the test method.* A predetermined amount of the compound is dissolved in an inorganic medium (mineral nutrient solution), providing a usual concentration of 2 mg active substance per liter (AS/l). The solution is inoculated with a small number of micro-organisms from a mixed population and kept in closed bottles in the dark in a constant temperature bath at 20±1 °C. The degradation is followed by oxygen analyses over a 28-day period. A control with inoculum, but without test material, is run parallel for the determination of oxygen blanks.

(v) *Quality criteria*—(A) *Reproducibility.* The reproducibility of the method is appropriate for a screening test for "ready biodegradability," which has solely an acceptance function, but is not sufficient for a final decision regarding biodegradibility.

(B) *Sensitivity.* A starting concentration of 2 mg AS/l usually allows the determination of 95 percent degradation. Compounds with a low TOD may require higher starting concentrations.

(C) *Specificity.* The test is applicable for the biodegradability evaluation of organic compounds. Most experience has been gathered with water soluble compounds; however, the test lends itself, at least in principle, to the biodegradability evaluation of volatile and insoluble compounds.

(D) *Possibility of standardization.* The test is suited for standardization.

(E) *Possibility of automation.* Although the whole test cannot be automated, parts of it, e.g., the analysis, may. The procedure is well suited, however, for being operated with whole series of test materials.

(2) *Description of the test procedure*—(i) *Preparations*—(A) *Reagents*—(*1*) *Distilled or ion exchange water*. Containing not more that 0.01 mg Cu/l, air saturated. Volume according to the need of the day, e.g., 50 l, at room temperature, as close as possible to 20 °C, aerated strongly for 20 minutes with compressed air. Generally, the water is ready for use after standing for 20 hours at 20 °C. Oxygen is determined for control purposes. The concentration at 20 °C should be about 9 mg $O_2$/l. All transfer and filling operations of the air saturated water must be conducted bubble-free by siphon.

(*2*) *Standard dilution water for BOD determinations according to H5 of DEV* (see paragraph (d)(1) of this section).

(*i*) Solution 1: 8.5g of $KH_2PO$, 21.75g of $K_2HPO_4$, 33.3g of $Na_2HPO_4 \cdot 2H_2O$, and 1.7g of $NH_4Cl$ are dissolved in 500 ml of distilled water. The solution is made up to 1 l. The pH ought to be 7.2.

(*ii*) Solution 2: 22.5 g of $MgSO_4 \cdot 7 H_2O$/l.

(*iii*) Solution 3: 27.5 g of $CaCl_2$/l.

(*iv*) Solution 4: 0.25 g of $FeCl_3 \cdot 6 H_2O$/l.

1 ml each of solutions 1 to 4 are added to 1 litre of aerated distilled water.

(B) *Materials*. (*1*) Calibrated 250–300 ml BOD bottles with glass stoppers (Uncalibrated narrow neck 250 ml bottles with glass stoppers whose volumes have been determined may be used instead without causing any appreciable error.) All glassware is cleaned according to the H23 procedure under paragraph (d)(2) of this section.

(*2*) Several 2, 3 and 5-l bottles with self-made litre marks for the preparation of the experiments and for the filling of the BOD bottles.

(*3*) Pipettes of 1 to 10 ml volume.

(*4*) Funnels and coarse filter paper.

(*5*) Baby bottles for the preparation and shaking of the inoculum.

(*6*) Water bath for keeping the bottles at constant temperature under the exclusion of light.

(C) *Inoculation*. The following inocula may be used:

(*1*) An aqueous suspension of unfertilized garden soil. 100g of unfertilized garden soil (soil from a greenhouse which is at constant temperature throughout the year is especially advantageous) are dispersed in chlorine-free tap water (1 liter). After 30 minutes the suspension is filtered through a coarse filter and the first 200 ml of the filtrate are discarded. The main part of the filtrate serves for the inoculation (1 drop from a pointed pipette per liter of final volume, see Procedure, below). The inoculum is prepared immediately before the experiment. It must be aerated if it is to be kept for several hours. The number of bacteria may be determined according to the pour plate method or with nutrient pad sets. There should be no more than $10^3$ to $10^5$ bacteria per milliliter of final volume.

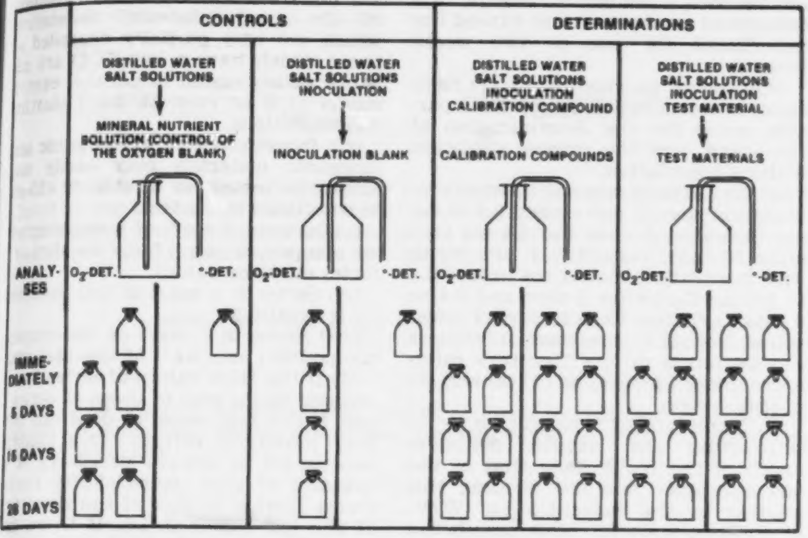
(*2*) Effluent from an activated sludge plant or trickling filter. Inoculation should preferably be made using a secondary effluent of good quality collected from a treatment plant dealing with predominantly domestic sewage. The effluent must be kept under aerobic conditions in the period between sampling and application. To prepare the inoculum the sample is filtered through a coarse filter, the first 200 ml being discarded. The rest of the filtrate is kept aerobic until used. The inoculum must be used on the day of collection.

(*3*) Effluent from a strongly aerated laboratory activated sludge plant. The inoculum is prepared as described in previous section.

(*4*) Composite inoculum. Equal volumes of the three inoculum samples are combined, mixed well and the final inoculum drawn from this mixture. The suitability of the inoculum is checked by means of a control substance.

(ii) *Procedure*. (A) (*1*) Direct comparisons are always necessary in biological experiments. Therefore groups of parallel bottles are prepared for the determination of the BOD of the test and control substances in simultaneous experimental series, see the following Figure 1:

FIGURE 1—SCHEME FOR THE BOTTLE ARRANGEMENT FOR THE CLOSED BOTTLE TEST


CONTROLS
DETERMINATIONS
DISTILLED WATER SALT SOLUTIONS
MINERAL NUTRIENT SOLUTION (CONTROL OF THE OXYGEN BLANK)
DISTILLED WATER SALT SOLUTIONS INOCULATION
INOCULATION BLANK
DISTILLED WATER SALT SOLUTIONS INOCULATION CALIBRATION COMPOUND
CALIBRATION COMPOUNDS
DISTILLED WATER SALT SOLUTIONS INOCULATION TEST MATERIAL
TEST MATERIALS
ANALYSES
$O_2$-DET.
*-DET.
IMMEDIATELY
5 DAYS
15 DAYS
28 DAYS

(* = SPECIFIC ANALYSIS IF AVAILABLE)

If chemical analyses are performed simultaneously a sufficient number of bottles—including the controls for the inoculum and the blank—have to be prepared [e.g., 7 or 15 parallel bottles are prepared for one test material for the 0, 5, 15 and 28-day tests after a sufficient volume of water has been prepared in large bottles (see Reagents above)].

(*2*) These large bottles are first filled o about one third of their volume with distilled water by hose. Then the ndividual salt stock solutions are pipetted into these bottles according to he final volume, and the respective est or control materials are added in uch amounts that final concentraions of 2 and sometimes 5 or 10 mg/l higher concentrations only in case of oorly degradable compounds and hose with a low TOD) are attained ee General considerations below). ubsequently the experimental soluon is inoculated with 1 drop (from a pointed pipette) per liter of final volume, and the blank is inoculated similarly. Finally, the solutions are made up to volume with a hose which reaches down to the bottom of the flask, which achieves adequate mixing.

(*3*) Subsequently each prepared solution is filled immediately into the respective group of bottles by hose from the lower quarter (not from the bottom!) of the bottle. Furthermore, the zero controls are analyzed or preserved for the later analyses (for the $O_2$-determination by precipitation with $MnCl_2$ and NaOH; for the surfactant determination by preservation with 50 mg/l of $HgCl_2$). The remaining parallels are placed in a water bath at 20 °C, kept in the dark, and removed after 5, 15 and 28 days, respectively, from the bath, and analyzed. The result is recorded as percentage biodegradability and should be visualised as a diagram.

(*4*) The test can be finished before the 28th day in the case where a plateau is observed before the 28th day. In the case where a degradation has obviously started on day 28 but has not reached a plateau on day 28, it is considered good practice to extend the experiment for one or two weeks longer.

(B) *General considerations.* (*1*) Each series is accompanied by complete parallel series for the determination of the blank, and the oxygen depletion without inoculation.

(*2*) The oxygen depletion without inoculation should not exceed 0.2 to 0.3 mg $O_2$/l after 5 days and 0.4 mg $O_2$/l after 28 days, respectively; the blank with inoculation should not exceed 0.4 to 0.5 mg $O_2$/l after 5 days and 0.5 to 0.6 mg $O_2$/l after 15 to 28 days. Typical values for the reference substances can be found in the literature references under paragraphs (d) (8) and (9) of this section.

(*3*) Degradability is calculated by (*i*) subtracting the oxygen depletion result of the blank from that of the test substance and (*ii*) dividing this number by the concentration (W/V) of the test material. This calculation gives the oxygen depletion in mg$O_2$/mg of active material; this value is transformed to the "% biodegradability" according to Data and reporting under paragraph (c) of this section for the day under consideration.

(*4*) If the structure or elemental composition of the test material is unknown, or it is a mixture of unknown organic compounds, the chemical oxygen demand (COD) may serve as a reference instead of the TOD.

(*5*) The concentration of about 9 mg of dissolved oxygen per liter of dilution water at 20 °C limits the possible starting concentration of the test material to about 2 mg/l in order to guarantee that an oxygen concentration of 4–5 mg/l remains after the oxidation of the test substrate. Substances which are only partially degraded or those which have a low TOD are advantageously tested in parallel experiments at 5 or even 10 mg/l starting concentrations.

(C) *Inhibition test.* (*1*) Organic and inorganic materials may easily and simply be tested for inhibitory effects in the closed bottle test:

(*i*) Series 1: 2 mg/l of a well-degraded compound (e.g., fatty alcohol+10 moles ethylene oxide).

(*ii*) Series 2: × mg/l of test material (× is usually 2).

(*iii*) Series 3: 2 mg/l of the control compound plus × mg/l of test material.

(*2*) If the BOD values of series 3 correspond to the sum of those of series 1 and 2, the test material does not display inhibitory effects. This control experiment is always necessary if a negative or poor degradability result seems illogical in view of the structure of the test material, i.e., if there are indications that it may be caused by inhibition.

(iii) *Analytical means.* (A) Oxygen determination according to Winkler under paragraph (d)(3) of this section or electrometrically, if suitability is assured.

(B) In case of anionic surfactants: MBAS-determination according to H23 of DEV under paragraph (d)(2) of this section.

(C) In case of nonionic surfactants: BiAS(bismuth active substance)-determination according to Wickbold under paragraph (d)(4) of this section.

(c) *Data and reporting*—(1) *Treatment of results*—(i) *Calculation of the theoretical oxygen demand (TOD).* (A) The TOD of the compound $C_cH_hCl_{cl}N_nNa_{na}O_oP_pS_s$ of the molecular weight is calculated according to:

$$\mathrm{TOD}_{NH3} = \frac{16\,[2c + (h - cl - 3n)/2 + 3s + 5p/2 + na/2 - o]}{\text{mol. wt.}}$$

(B) This calculation implies that C is mineralised to $CO_2$, H to $H_2O$, P to $P_2O_5$ and Na to $Na_2O$. Halogen is eliminated as hydrogen halide and nitrogen as ammonia.

Example: Glucose $C_6H_{12}O_6$, mol. wt. = 180

$$TOD = \frac{16(2 \cdot 6 + 12/2 - 6)}{180} = 1.07 \text{ mg } O_2\text{/mg active substance}$$

(C) Molecular weights of salts other than those of the alkali metals are calculated on the assumption that the salts have been hydrolyzed.

(D) Sulfur is schematically oxidized to the state of +6. Example: Alkylbenzenesulfonate, LAS,$C_{18}H_{29}SO_3Na$, mol.wt. = 348

$$TOD = \frac{16(36 + 29/2 + 3 + 1/2 - 3)}{348} = 2.34 \text{ mg } O_2\text{/mg active substance}$$

(E) In the case of nitrogen-containing compounds the nitrogen may be eliminated as ammonia, nitrite, or nitrate corresponding to different theoretical oxygen demands:

$$TOD_{NO2} = \frac{16\,[2c + (h\text{-}cl)/2 + 3s + 3n/2 + 5p/2 + na/2 - o]}{\text{mol. wt.}}$$

$$TOD_{NO3} = \frac{16\,[2c + (h\text{-}cl)/2 + 3s + 5n/2 + 5p/2 + na/2 - o]}{\text{mol. wt.}}$$

(F) Suppose full nitrate formation would have been observed by analysis in ase of a secondary amine:

$(C_{12}H_{25})_2$ NH; mol. wt. = 353

$$TOD_{NO3} = \frac{16(48 + 51/2 + 5/2)}{353} = 3.44 \text{ mg } O_2\text{/mg active substance}$$

(ii) *Calculation of results.* For the calculation of the percentage biodegradabi-ty after n days the determined oxygen depletions are divided by the concentra-ion (W/V) of active substance.

Then—

$$\text{Percent biodegradability} = \frac{\text{mg}O_2\text{/mgAS}}{TOD} \times 100$$

or

$$\text{Percent biodegradability} = \frac{\text{mgO}_2/\text{mgAS}}{\text{mgCOD/mgAS}} \times 100$$

(2) *Test report.* The raw data may be listed in and evaluated according to the following form sheet:

(3) *Interpretation of results.* See paragraph (a) of this section.

### SAMPLE FORM SHEET FOR THE CLOSED BOTTLE TEST

Exp. No ........ ______

Date of start of test ........ ______

Test/standard material ........ ______

Theoretical test concentration. mgAS/l.

Inoculum ........ ______

### SAMPLE FORM SHEET FOR THE CLOSED BOTTLE TEST—Continued

Analysis: (Winkler method or oxygen electrode). ______

TOD or COD of test material. mgO₂/mgAS.

Temp. of the dilution water after aeration. K (O$_C$).

O₂-conc. of the distilled (deionised) water after aeration and standing before start of test. mg O₂/l.

PART I: O₂ Determinations:

| Culture medium | Flask No. | Analyses | mgO₂/l after *x* days | | | |
|---|---|---|---|---|---|---|
| | | | 0 | 5 | 15 | 28 |
| Mineral nutrient solution *without* test material and *without* inoculum. | O₂ ........ | | | | | |
| | Control ........ | $c_1$ | | | | |
| | | $c_2$ | | | | |
| | Mean ........ | $m_C = \frac{c_1 + c_2}{2}$ | | | | |
| Mineral nutrient solution *without* test material but *with* inoculum. | 1 ........ | $c_3$ | | | | |
| | 2 ........ | $c_4$ | | | | |
| | Mean blank ........ | $m_b = \frac{c_3 + c_4}{2}$ | | | | |
| Mineral nutrient solution *with* test material and *with* inoculum. | 1 ........ | $a_1$ | | | | |
| | 2 ........ | $a_2$ | | | | |
| | Mean test material ........ | $m_t = \frac{a_1 + a_2}{2}$ | | | | |

PART II: O₂ Depletions (mg BOD/l) after *x* days:

$$\text{BOD}_x = (m_0 - m_{tx}) - (m_0 - m_{bx})$$

(This difference is important as a check for the validity of the test.)

| mg BOD/l after *x* days | | |
|---|---|---|
| 5 | 15 | 28 |
| | | |

PART III: Evaluation:

$$\text{Percent biodegradability (I)} = \frac{\text{mg BOD}_x/\text{l}}{\text{mg AS/l.TOD}} \cdot 100$$

or

$$\text{Percent biodegradability (II)} = \frac{\text{mg BOD}_x/\text{l}}{\text{mg AS/l.COD}} \cdot 100$$

| | After x days | | |
|---|---|---|---|
| | 5 | 15 | 28 |
| | | | |
| | | | |
| | | | |

d) *Literature references.* For addi-
nal background information on this
t guideline the following references
ould be consulted:

1) "Methode H5," *Deutsche Ein-*
*tsverfahren zur Wasseruntersu-*
*ng,* (Verlag Chemie: Weinheim,
0).

2) "Methode H23," *Deutsche Ein-*
*tsverfahren zur Wasseruntersu-*
*ng,* (Verlag Chemie: Weinheim,
0).

) "Methode G2," *Deutsche Ein-*
*tsverfahren zur Wasseruntersu-*
*ng,* (Verlag Chemie: Weinheim,
)).

) Wickbold, R. *Wasser,* 33: 229,
6).

) Wickbold, R. *Tenside Detergents*
(1971).

) "Oxygen Demand," *Standard*
*hods for the Examination of Water*
*Wastewater,* 12 Ed. American
lic Health Association, American
er Works Association Water Pollu-
Control Federation, (1965) p. 510.

Fisher, W.K., Fette, Seifen, *An-*
*hmittel,* 65: 37-32 (1963).

Fischer, W.K., Gerike, P. and
nid, R., *Wasser- und Abwasserfors-*
*g* 7: 99-118 (1974).

Gerike, P. and Fischer, W.K.,
*oxicology & Environmental*
*y,* Vol. *3,* No. 2, 159-173 (1979).

R 39485, Sept. 27, 1985]

### 3220 Ready biodegradability: Modi- ed MITI test (I).

*Introductory information*—(1)
*equisites.* (i) An analytical method
be available for determining the
entration of the test material in
est solution.

The empirical formula of the test
rial is required so that the theo-
l oxygen demand (TOD) may be
lated.

*Guidance information.* (i) Infor-
on on the relative proportions of
the major components of the test material will be useful in interpreting the results obtained, particularly in those cases where the result lies close to the "pass level."

(ii) Information on the toxicity of the chemical may be useful to the interpretation of low results and in the selection of appropriate test concentrations.

(3) *Interpretation of results.* Because of the stringency of this test, a result of less than 60 percent of BOD (biochemical oxygen demand) (70 percent LPC (loss of parent compound)) (within 28 days) does not necessarily mean that the test compound is not biodegradable under environmental conditions, but indicates that more work will be necessary to establish biodegradability. The possibility that nitrogen-containing compounds may affect the results should be considered.

(4) *Qualifying statements.* (i) The method is only applicable to those organic test materials which, at the concentration used in the test:

(A) Have negligible vapor pressure.

(B) Are not inhibitory to bacteria.

(C) Do not reach and react with the $CO_2$ adsorbant.

(ii) This test has been found suitable by the OECD Expert Group "Degradation/Accumulation" for determining the "ready biodegradability of organic chemicals under aerobic conditions." It has been tested in the OECD Laboratory Intercomparison Test Program (1978-1980).

(5) *Recommendations.* (i) Test chemicals giving a result of greater than 60 percent BOD, 70 percent LPC, (within 28 days) should be regarded as readily biodegradable.This level must be reached within 10 days of biodegradation exceeding 10 percent.

(ii) If the test material is not soluble at the test concentration, special measures, such as the use of ultrasound dispersion, may have to be employed to achieve a good dispersion of the test material.

(6) *Standard documents.* This Test guideline was based upon the order prescribing the items of test, relating to new chemical substances of the Chemical Substance Control Law (Japanese Law No. 117, 1973). Order of the

Japanese Prime Minister, the Minister of Health and Welfare, and the Minister of International Trade and Industry, No. 1 (July 13, 1974).

(b) *Method*—(1) *Introduction, purpose, scope, relevance, application and limits of test.* (i)(A) Purpose: Measurement of biochemical oxygen demand and the analysis of residual chemicals, intermediates necessary in the evaluation of the biodegradability of chemical substances.

(B) Appropriate chemicals: non-volatile and water soluble (more than 100 ppm) organic chemicals.

(C) Applicable chemicals: slightly water soluble chemicals; volatile chemicals (modified BOD meter should be used) ($C_{water}/C_{air}>1$ (C=concentration))

(D) Non-applicable chemicals: volatile chemical substances ($C_{water}/C_{air}<1$).

(ii) Definitions and units—(A) *Definitions.* (*1*)

(*i*) Percentage degradation $= \dfrac{\text{BOD-B}}{\text{TOD}} \times 100\%$

or

(*ii*) Percentage degradation $= \dfrac{\text{Sb-Sa}}{\text{Sb}} \times 100(\%)$

(*2*) BOD: Biochemical oxygen demand (experimental) (mg) of the test compound measured on the BOD curve.

(*3*) B: Oxygen consumption (experimental) (mg) of basal culture medium to which the inoculum is added measured on the BOD curve.

(*4*) TOD: Theoretical oxygen demand (theoretical) (mg) required when the test compound is completely oxidised.

(*5*) Sa: Residual amount (experimental) (mg) of the test compound after completion of the biodegradability test.

(*6*) Sb: Residual amount (experimental) (mg) of the test compound in the blank test with water to which only the test compound has been added.

(B) [Reserved]

(iii) *Reference substances.* In some cases when investigating a new substance reference substances may be useful; however, specific reference substances cannot yet be recommended. In order to check the activity of the inoculum, the use of control substances is desirable. Aniline, sodium acetate or sodium benzoate may be used for this purpose.

(A) *Aniline.* If the percentage of degradation of aniline calculated from the oxygen consumption does not exceed 40 percent after 7 days or 65 percent after 14 days, the test is regarded as invalid.

(B) [Reserved]

(iv) *Principle of the test method.* This test method is based on the following conditions:

(A) Test chemicals as sole organic carbon sources, and

(B) No adaptation of micro-organisms to test chemicals.

An automated closed system oxygen consumption measuring apparatus (BOD meter) is used. Chemicals to be tested are inoculated with micro-organisms in the testing vessels. During the test period, biochemical oxygen demand is measured continuously by the BOD meter. Biodegradability is calculated on the basis of BOD and supplemental chemical analysis is undertaken, such as measuring dissolved organic carbon concentration, concentration of residual chemicals, etc.

(v) *Quality criteria*—(A) *Reproducibility.* Generally good, especially so for chemicals of water solubility greater than 0.1 g/l.

(B) *Sensitivity.* (*1*) Oxygen consumption: Detection limit=1 mg (oxygen consumption by micro-organisms)

(*2*) Chemical analysis: Depends on the sensitivity of analytical methods.

(C) *Specificity.* Applicable to every kind of chemical, for which $C_{water}/C_{air}>1$. For volatile chemicals a "modified BOD meter," composed of capillary tubing and normal BOD meter, should be used under paragraph (b)(3) of this section.

(D) *Possibility of automation.* By using a BOD meter under paragraph (b)(3) of this section oxygen consumption by micro-organisms (in a closed system) is recorded automatically.

(2) *Description of the test procedure*—(i) *Preparations*—(A) *Apparatus.* BOD meter equipped with 6 bottles 300 ml each):

(*1*) Bottles 1 and 2: deionized water Distilled water must never contain ore than 10 percent of organic arbon introduced by the test sub- tance), 300 ml+test chemical, 30 mg.

(*2*) Bottles 3 and 4: basal culture edium, 300 ml+activated sludge, 9 g (dry base)+test chemical, 30 mg.

(*3*) Bottle 5: basal culture medium, 0 ml+activated sludge, 9 mg (dry se)+aniline, 30 mg.

(*4*) Bottle 6: basal culture medium, 0 ml+activated sludge, 9 mg (dry se).

B) *Pretreatment of test chemical.* ) Where the test compound is not luble in water up to the test concentration, the test compound pulverized finely as possible is employed.

2) Where the test compound is vola- e, test chemicals should be well oled to prevent evaporation.

3) If necessary, identification of the t sample should be undertaken.

C) *Basal culture medium.* To each 3 of solution A, solution B, solution C d solution D, water is added to make to 1000 ml. (Deionized water is used roughout.)

*1*) *Solution A.* 21.75 g of dipotas- m hydrogen phosphate, 8.5 g of po- sium acid phosphate, 44.6 g of diba- sodium phosphate dodecahydrate d 1.7 g of ammonium chloride are solved in water and the volume is de up to 1000 ml. (The pH of the ution is 7.2.)

(*2*) *Solution B.* 22.5 g of magnesium sulfate heptahydrate is dissolved in water and the volume is made up to 1000 ml.

(*3*) *Solution C.* 27.5 g of calcium chloride is dissolved in water and the volume is made up to 1000 ml.

(*4*) *Solution D.* 0.25 g of ferric chloride hexahydrate is dissolved in water and the volume is made up to 1000 ml.

(D) *Activated sludge*—(*1*) *Sludge sampling sites.* Sludge sampling is made, in principle, at not less than 10 places throughout the country, chiefly in those areas where a variety of chemical substances may be considered to be consumed and discarded. For example, standard activated sludge of the Japanese Chemical Biotesting Center is taken up from the following places and mixed:

(*i*) *City sewage plant.* 3 plants located in the northern, central and southern part of Japan.

(*ii*) *Industry sewage plant.* One plant used for the waste water treatment of chemical industries.

(*iii*) *River.* 3 rivers located in the northern, central and southern parts of Japan.

(*iv*) *Lake.* One lake located in the middle of Japan.

(*v*) *Sea.* 2 inland seas of Japan.

(*2*) *Frequency of sludge sampling.* Sludge sampling should be made, in principle, four times a year, in March, June, September and December.

(*3*) *Sludge sampling methods.* (*i*) City sewage: 1 liter of return sludge at a sewage disposal plant.

(*ii*) Rivers, lakes and marshes or sea: 1 liter of surface water and 1 liter of surface soil on the beach which is in contact with atmosphere.

(*4*) *Preparation.* The sludge samples collected from the sampling sites are mixed by stirring in a single container, and the mixture is allowed to stand. The floating foreign matters are removed, and the supernatant is filtered with No. 2 filter paper. The filtrate is adjusted to pH 7.0±1.0 with sodium hydroxide or phosphoric acid, transferred into a culture tank and aerated.

(*5*) *Culture.* Thirty minutes after stopping the aeration of the solution obtained above, approximately ⅓ of the whole volume of the supernatant is removed. An equal volume of 0.1

percent synthetic sewage (0.1 percent synthetic sewage: 1 g of glucose, 1 g of peptones and 1 g of monopotassium phosphate are dissolved in 1 liter of water and the solution is adjusted to pH 7.0±1.0 with sodium hydroxide) is added to the remaining portion of the supernatant and the mixture is aerated again. This procedure is repeated once every day. The culturing is carried out at 25±2 °C.

(*6*) *Control.* For the control of the culturing step, the following items are checked and necessary adjustments are made.

(*i*) Appearance of supernatant: The supernatant of active sludge should be clear.

(*ii*) Precipitability of active sludge: The active sludge, being in large flocks, must have high precipitability.

(*iii*) State of formation of active sludge: In the case where growth of flocks is not observed, either the volume of 0.1 percent synthetic sewage to be added or the frequency of addition of synthetic sewage is increased.

(*iv*) The pH of the supernatant is 7.0±1.0.

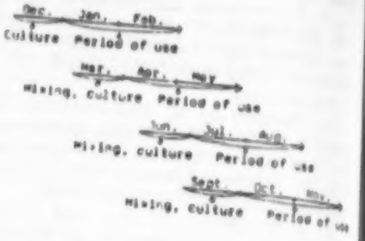
(*v*) Temperature: The temperature for cultivation of active sludge is 25±2 °C.

(*vi*) Amount of aeration: In replacing the supernatant with the synthetic sewage, the solution in the culturing tank must be sufficiently aerated to maintain the dissolved oxygen concentration of the solution above 5 ppm.

(*vii*) Microflora of activated sludge: When the active sludge is microscopically observed (at 100~400×magnification), a number of protozoa of different species together with cloudy flocks must be seen.

(*viii*) Mixing of fresh and old activated sludge: In order to maintain fresh and old activated sludges at the same activity, the filtrate of the supernatant of an activated sludge in use in the test is mixed with an equal volume of the filtrate of the supernatant of an activated sludge freshly collected and the mixture is cultured.

(*ix*) Checking of activity of activated sludge: Activity of activated sludge should be checked periodically, at least once every three months, with standard substances by applying the test method provided below. Where fresh and old activated sludge samples are mixed, especially, careful checking must be done in relation to the old activated sludge. Examples of preparation of activated sludge samples and period of use:



(The same pattern of preparation and use follows.)

(E) *Addition of test compound and preparation for test.* The following test vessels are provided and adjusted to the test temperature.

(*1*) A test vessel containing the basal culture medium, to which is added 100 ppm (W/V) of test compound: the pH of this solution is adjusted to 7 before the inoculation of active sludge, if necessary.

(*2*) A test vessel for the control blank test, containing only the basal culture medium.

(*3*) A test vessel containing water to which is added 100 pm (W/V) of the test compound.

(*4*) A test vessel containing basal culture medium to which is added 100 ppm (W/V) of aniline or any other of the control substances.

(F) *Inoculation of active sludge.* Inoculum is added to the test vessels 1, 2 and 4 above so that the suspended matter defined in the Japanese Industrial Standards described under paragraph (b)(4) of this section is contained at a concentration of 30 ppm (V/V).

(ii) *Test conditions.* (A) Concentration of test chemicals: 100 ppm (W/V).

(B) Concentration of activated sludge: 30 ppm (W/V).

(C) Test temperature: 25 °C ±2 °C.

(D) Period: 14 days.

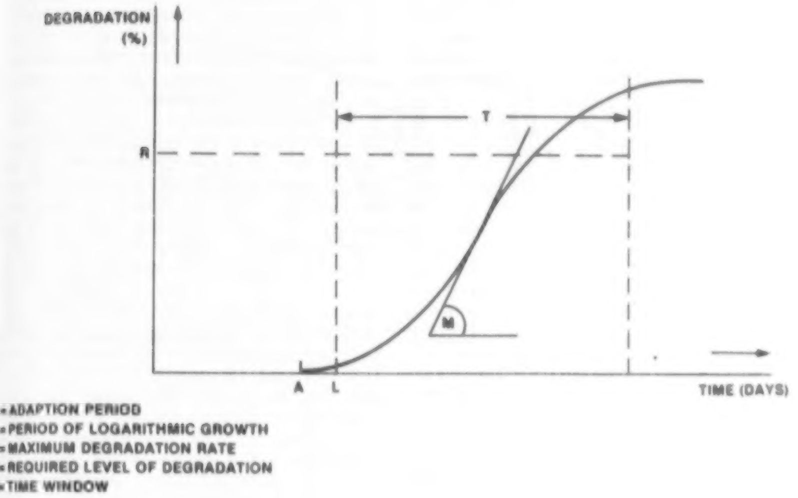
(E) Perform in darkness. Every day, the temperature, the change in color of the culturing vessel should be checked.

(F) Stir vigorously with mechanical stirrer.

(iii) *Performance of the test.* (A) The BOD curve is obtained continuously and automatically for 14 days.

(B) After the 14-day testing period, pH, residual chemicals, and intermediates in the testing vessels are analysed.

FIGURE 1—DEGRADATION CURVE OF A READILY DEGRADABLE COMPOUND



The test chemicals in the testing sel without activated sludge are o analyzed in order to confirm ether there is any change in the t chemical during the testing period any loss of the original test chemi- by evaporation or adsorption by walls of test vessels, etc.

v) *Analytical means.* (A) If the test npound is soluble in water, the re- ual amount of total organic carbon lso determined.

) Where a total organic carbon an- zer is used: 10 ml of the tested solu- n is sampled from the test vessel l centrifuged at 3000 g for 5 min- s. The residual amount of the total anic carbon in the supernatant is determined on a total organic carbon analyzer.

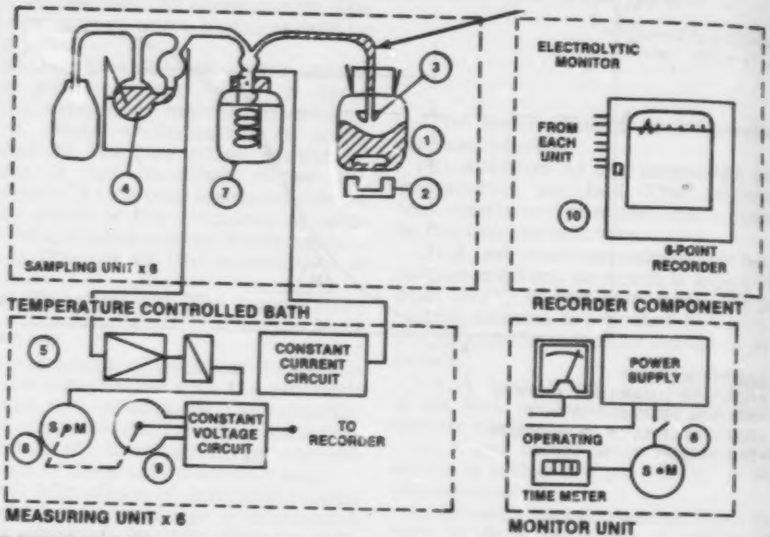
(*2*) Where other analyzers are used: The total content of a test vessel is extracted with a suitable solvent for the test compound, and, after proper pretreatment such as concentration, the residual amount of the test compound is determined on an analyzing instrument (gas chromatography, absorptiometry, mass spectrometry, atomic absorption spectrophotometry, etc.).

(B) For volatile chemicals, the temperature control bath of the BOD meter should be cooled to 10 °C and this temperature held for at least 30 min, in order to prevent evaporation.

The analytical procedures (a) and (b) should then be started.

(3) *Principle of closed system oxygen consumption measuring apparatus.* (i) The coulometer is an instrument for measurement of the oxygen consumption of micro-organisms using electrochemical analysis process (coulometry).

(ii) The following is a block diagram. The BOD meter is produced by Ohkura Electric Co., Ltd., 1-11-14, Shibuya, Shibuya-ku, Tokyo, Japan. (For volatile chemicals, a capillary tube should be installed between each testing vessel and electrolytic bottle.) (For modified BOD meter, hatched part of tube should be replaced with capillary tube.)


ELECTROLYTIC MONITOR
FROM EACH UNIT
6-POINT RECORDER
RECORDER COMPONENT
SAMPLING UNIT x 6
TEMPERATURE CONTROLLED BATH
CONSTANT CURRENT CIRCUIT
S M
CONSTANT VOLTAGE CIRCUIT
TO RECORDER
MEASURING UNIT x 6
POWER SUPPLY
OPERATING
TIME METER
S M
MONITOR UNIT
1
2
3
4
5
6
7
8
9
10

THE SPECIMEN CONTAINED IN CULTIVATING BOTTLE (1) IS STIRRED BY MEANS OF A MAGNETIC STIRRER (2). WHEN THE REACTION PROGRESSES, THE DISSOLVED OXYGEN IN LIQUID WILL BE CONSUMED. OXYGEN ($O_2$) IN THE SPACE IN THE CULTIVATING BOTTLE IS DISSOLVED IN LIQUID, RESULTING IN GENERATION OF $CO_2$ IN ITS PLACE.

(A) The specimen contained in cultivating bottle (1) is stirred by means of a magnetic stirrer (2). When the reaction progresses, the dissolved oxygen in liquid will be consumed. Oxygen ($O_2$) in the space in the cultivating bottle is dissolved in liquid, resulting in generation of $CO_2$ in its place.

(B) As this $CO_2$ is absorbed by soda lime (3), the partial pressure of oxygen in the space and the total pressure decrease.

(C) The drop in pressure is detected and converted into an electric signal by means of an electrode type manometer (4) and is amplified by an amplifier (5) for operating a relay circuit (6), resulting in operation of a synchronous motor (8). Simultaneously, by constant current, electrolytic oxygen is generated from sulfuric acid copper solution contained in an electrolytic bottle (7).

(D) This oxygen is supplied to the cultivating bottle and restoration of pressure is detected by means of the manometer, resulting in switching off of the relay circuit and stopping the electrolytic and synchronous motor.

(E) The upper space in the cultivating bottle is always kept under a constant pressure of oxygen and the quantity of oxygen consumed in the cultivating bottle is proportional to the quantity of electrolytic oxygen. As this quantity of electrolytic oxygen is proportional to electrolytic time, there is a constant electrolysis current. Accordingly, the revolution angle of a synchronous motor (9) is converted to an mV signal by means of the interlocking potentiometer, resulting in an indicator quantity of consumed oxygen at the recorder (10).

(4) *Suspended matter.* (i) (From Japanese Industrial Standards K0102-10.2.)

(A) Suspended matter is that material which can be separated by filtration or by means of a centrifugal separator. It can be determined by any of the methods described below. When the test water is difficult to filter the centrifugal separation method should be applied; when the test water contains an extremely large quantity of suspended matter, the Büchner funnel method should be used.

(B) Test water is taken from the waste water passed through a 2 mm mesh sieve. At least 5 mg of the filtrate are necessary for the determination.

(ii) *Filtration through filter paper—(A) Sintered glass filter method—(1) Apparatus—(i) Sintered glass filter.* A crucible-type sintered glass filter IG2 or a Büchner funnel-type sintered glass filter 3G2.

(*ii*) [Reserved]

(*2*) *Procedure.* Prepare two sintered glass filters of the same type and of approximately the same weight; lay six sheets of filter paper in them and pour water through several times so that they adhere by suction. Then transfer the filters to an air oven and dry them for two hours at 105–110 °C. Allow them to cool in a desiccator, and weigh. (When a chemical balance is used, the lighter filter may be used as a supplementary weight.) Pour a suitable amount of the test water into the heavier filter (take sufficient test water to give a weight of suspended matter of not more than 5 mg after drying. Ordinarily, 200 ml of the test water is enough. However, if the test water is difficult to filter, 10 ml from each test water sample must be added from the 10 ml measuring cylinder during the filter process.), filter it by suction, and wash the wall of the filter several times with the filtrate, in order to remove substances adhering to the wall. Next, pour the filtrate into the lighter filter several times and filter it by suction. Dry the two filters in the air oven for two hours at 105–110 °C, and allow them to cool in a desiccator. Weigh each filter (when a chemical balance is used, the lighter filter may be used as a supplementary weight), obtain the difference in weight before and after the filtration, and calculate the quantity of the suspended matter in ppm according to the following formula:

$$S=(a-b)\times \frac{1000}{V}$$

where:

S: Suspended matter (ppm)

a: Difference in weight before and after the filtration of test water (mg)

b: Difference in weight before and after the filtration of the filtrate (mg) (when a chemical balance is used, b=0)

V: Amount of test water (ml)

(*3*) *Remarks.* (*i*) To determine the ignition loss of volatile suspended matter, a test should be carried out in accordance with the Glass Fiber Paper Method under paragraph (b)(4)(ii)(A)(*3*) (*iii*) of this section, or, after washing the suspended matter together with filter paper into a crucible or an evaporating dish, dry and ignite in muffle furnace.

(*ii*) When the soluble evaporated residue is less than 5000 ppm, correction (for difference in weight of the filtrate before and after filtration) may be omitted. However, when a chemical balance is used, the lighter filter should be used as a supplemental weight so the filtration of the filtrate can be carried out at the same time. Even when a direct reading balance is used the weight varies with the hygroscopic properties of substances contained in the test water, and with other conditions, so it is desirable that a correction be performed by obtaining the blank test value of the filter through which the filtrate is passed. In the case of the test water containing fats and oils, grease, wax, etc., a portion of these substances should be determined as the suspended matter. When the determination of the suspended matter exclusive of oils and fats is required, pour 10 ml volumes of n-hexane several times through the filter which has been dried and weighed after filtration and wash out the fats and oils. Then dry the filter and weigh.

(*iii*) Glass Fiber Paper Method (GFP Method): Fix an appropriate GFP (Whatman GF/B or equivalent) of known weight, which has been dried at 105 to 110 °C for 2 hours after washing, on a suitable supporting plate. Add the amount of test water to give a weight of the suspended matter after drying of over 5 mg. After filtration by suction, return a portion of the filtrate to the container holding the original test water. Wash down the suspended matter adhering to the walls of container and filter again on GFP by suction. Repeat this operation several times. Detach GFP from the filter and transfer it onto a water glass. Then operate as described for the Büchner funnel method under paragraph (b)(4)(ii)(B) of this section, and determine ppm of the suspended matter. After determination of the suspended matter determine the ignition residue of the suspended matter, if necessary, according to the operation described in the section on Filtration through asbestos layer, under paragraph (b)(4)(iii) of this section.

(B) *Büchner funnel method.* This method is applicable to samples containing a large quantity of suspended matter such as sludge.

(*1*) *Apparatus*—(*i*) *Perforated plate.* Stainless steel (SUS 27 or 28), approximately 0.5 mm in thickness, 50 mm or 90 mm in diameter. It is shaped like a watchglass with a slightly bent edge. Small holes about 0.5 mm in diameter are bored at suitable intervals all over its flat surface.

(*ii*) *Rubber packing.* A rubber ring 2 to 3 mm in thickness, 10 mm to 90 mm in diameter and about 10 mm in width, can be put in a Büchner funnel and can be used for filtration by suction with the perforated plate on it.

(*iii*) *Büchner funnel.* 50 mm or 90 mm.

(*2*) *Testing procedure.* (*i*) Prepare two perforated plates. Put rubber packing in Büchner funnel and place the perforated plate on it. Position the filter paper (grade 6), pour water on the filter paper several times and suck. Remove the filter paper with the perforated plates and dry at a temperature of 105 to 110 °C for 2 to 3 hours. Allow to cool in a desiccator and weigh to constant weight. (When chemical balance is used, the lighter perforated plate is used as supplemental weight.)

(*ii*) Next put the heavier perforated plate together with the filter paper in the funnel and filter 200 to 400 ml of the test water by suction. Pour the filtrate into the lighter plate with filter paper several times and continue as for first plate.

(*iii*) Obtain the difference in weight before and after this operation, and calculate ppm of the suspended substances contained in the test water by the following formula:

$$S = (a - b) \times \frac{1000}{V}$$

where:

S: Suspended substances (ppm)
a: Difference in weight before and after filtration of the test water (mg)
b: Difference in the weight before and after filtration of the filtrate (mg) (when chemical balance is used b=0)
V: Test water (ml)

See also Remarks under paragraph (b)(4)(ii)(A)(*3*) of this section.

(iii) *Filtration through asbestos layer*—(A) *Apparatus.* Gooch crucible, 25 to 35 ml.

(B) *Reagents.* Suspension of asbestos: add water to 15 g asbestos and, after removing fine portion by decantation several times, add water to make 1 liter.

(C) *Procedure.* (*1*) Prepare two Gooch crucibles (same shape and approximately same weight). After drying, pour about 20 ml of the well-stirred asbestos suspension to obtain a layer of asbestos about 3 mm thick (about 0.3 g) (when half the amount of asbestos solution is poured out put in the perforated plate and pour the other half of the solution) and suck gently.

(*2*) Next put the Gooch crucibles into the air oven. After drying for 2 hours at a temperature of 105 to 110 °C, allow to cool to constant weight in the desiccator and measure the weight of each crucible (when chemical balance is used, the lighter crucible is used as the supplemental weight). Attach the heavier crucible to the suction bottle and pour in enough test water to give a weight of suspended matter of more than 5 mg after drying and gently filter by suction. At this time, repeat the filtration of the initial portion of the filtrate.

(*3*) Next pour a small amount of filtrate into the lighter crucible several times using suction, then dry in the air oven for 2 hours at 105 to 110 °C, and allow to cool in a desiccator. Weigh the crucible and obtain the difference in weight using the crucible as a supplemental weight and calculate ppm of the suspended matter by the following formula:

$$S=(a-b)\times \frac{1{,}000}{V}$$

(See paragraph (b)(4)(ii)(B)(*iii*) of this section for clarification of symbols.)

(D) *Remarks.* The test water should be sampled as specified in glass filter method. When the soluble volatile residue is less than 5000 ppm, refer to remark 2 in the same section.

(iv) *Centrifugation method.* (A) This method is applicable to samples which are very difficult to filter due to their content of suspended matter.

(*1*) *Apparatus.* Centrifugal separator about 2000 rpm. Precipitation tube 50 to 100 ml.

(*2*) *Procedure.* (*i*) Pour into the precipitation tube enough test water to give more than 5 mg suspended matter.

(*ii*) After weighing each tube, centrifuge at about 2000 rpm for 20 minutes and precipitate the suspended matter in the test water. Remove the supernatant liquid by decantation. (When the determination of soluble evaporated residue is to be performed, keep the supernatant liquid).

(*iii*) Add 10 ml of the water to the precipitate, centrifuge again and remove the supernatant liquid by decantation.

(*iv*) Transfer the precipitate into an evaporating dish which has been previously heated to constant weight at 105 to 110 °C, and evaporate to dryness on the steam bath. After drying in the dryer at 105 to 110 °C, for 2 hours, allow to cool in a desiccator and weigh. (When a chemical balance is used, an evaporating dish of the same shape should be used as a supplemental weight after the blank test for it has been performed.) Obtain the difference in weight before and after this operation. Calculate ppm of the suspended matter by the following formula:

$$S=a\times \frac{1000}{V}$$

(See paragraph (b)(4)(ii)(B)(*2*) of this section for clarification of symbols.)

(*3*) *Remarks.* There should be a certain degree of difference in density between the dispersed phase and the dispersion medium to make centrifugal separation possible. When a particle of 1 mg is centrifuged at an angular velocity of w rad/sec at a position of r cm from the center of rotation, the centrifugal force which a particle receives is as follows:

(*i*) Supposing that the mass of the dispersion medium expelled by a particle is 1 mg

$$\text{then } F=(m-m')\,w^2r$$

(*ii*) Supposing that the specific centrifugal force is RCF and rotational frequency per minute is N (rpm) then $$\mathrm{RCF} = \frac{F}{(m-m')g} = \frac{w^2 r}{g} = 0.00001118\, rN^2$$

(*iii*) From the equation under paragraph (b)(4)(iv)(A)(*3*) of this section, it is clear that the centrifugal force near the surface differs from that at the bottom portion of the liquid. For instance, when N=2000 rpm and the distance between the surface of the liquid in the precipitation tube and the center of rotation is 5 cm (r=5 cm), RCF is 223 g; when the distance between the bottom of the precipitation tube and the central axis of rotation is 13 cm, RCF becomes 581 g. Therefore, the RCF value near the surface and that at the bottom should both be reported.

$$\text{Depth of the liquid layer} = \frac{(\text{RCF at the bottom}') - (\text{RCF at the surface})}{(\text{RCF at the bottom})} \times (\text{distance from the bottom})$$

In this test, a centrifugal separator whose bottom is 13 cm from the central rotation axis at a rotational frequency of 2000 rpm is regarded as standard.

(B) Calculation of suspended matter from the difference in weight of evaporated residue: Calculate the suspended matter from the difference between the total evaporated residue and the soluble evaporated residue.

A=B−C

where:

A=suspended matter (ppm)
B=total evaporated residue (ppm)
C=soluble evaporated residue (ppm)

(5) *Suspended matter formed at pH 7.* (From Japanese Industrial Standards K0102−10.3) For suspended matter formed when the test water is neutralized to pH 7±0.5.

(i) *Reagents.* (A) NaOH (sodium hydroxide) solution (4 to 24 w/v %)

(B) Acetic acid, diluted 1:2 to 1:16, acid:water.

(ii) *Procedure.* Place enough test water to give more than 5 mg suspended matter in a beaker and neutralize it with sodium hydroxide solution or with diluted acetic acid, according to the acidity/alkalinity of the test water, taking care to minimize the increase in the volume of the solution during neutralization. Then proceed according to procedures in the methods above to obtain amount of suspended matter at pH 7 and calculate ppm of the suspended matter formed at pH 7 by the following formula.

A=B−C (See paragraph (b)(4)(iv)(B) of this section for clarification of symbols.)

(iii) *Remarks.* (A) Depending on the kind of waste water, the weight of suspended matter may decrease when it is neutralized. In such cases, the weight of suspended matter should be reported as suspended matter formed at pH 7.

(B) Suspended matter formed at pH 7 may be determined with the supernatant liquid or filtrate after removing the suspended matter. This method is applicable to test water which contains a relatively small amount of suspended matter but which forms a large amount of precipitate after neutralization (without changing the first suspended matter) or to waste water which forms a relatively small amount of precipitate. The method should not be applied to waste water which is apt to cause the formation of a complex precipitate or a dissolution reaction by neutralization.

(c) *Data and reporting*—(1) *Treatment of results.* (i) Method for calculating the percentage degradation from the oxygen consumption.

$$\text{Percentage degradation} = \frac{\text{BOD-B}}{\text{TOD}} \times 100(\%)$$

(See Definitions and units, under paragraph (b)(1)(ii) of this section for clarification of symbols.)

(ii) Method for calculating the percentage degradation from the result of direct analysis.

$$\text{Percentage degradation} = \frac{\text{Sb}-\text{Sa}}{\text{Sb}} \times 100(\%)$$

See Definitions and units, under paragraph (b)(1)(ii) of this section for clarification of symbols.

(2) *Evaluation of results.* The following calculations are to be made:

(i) Calculation of theoretical oxygen demand.

| Element | Oxidised form |
|---|---|
| C | $CO_2$ |
| H | $H_2O$ |
| N | $NO_2$ |
| S | $SO_3$ |
| X(halogen) | X |

(ii) Recovery rate of analytical procedure.

(3) *Test report.* The test report should include the following points:

(i) *Information on the test chemicals.* Name, structural formula, molecular weight, purity, kind of impurities, physical chemical properties of test chemical, identification data of test chemical.

(ii) *Test conditions.* (A) Activated sludge: sludge sampling site and concentration.

(B) Test chemical: concentration.

(C) Test period.

(D) Test temperature.

(iii) *Analytical procedure.*

(A) Pretreatment.

(B) Analytical conditions of instrument.

(C) Recovery rate of analysis.

(D) Identification of intermediate.

(iv) *Results.* (A) BOD curves and instrument name.

(*1*) BOD (mg).

(*2*) B (mg).

(*3*) Sa (mg).

(*4*) Sb (mg).

(*5*) TOD (mg).

(B) Percentage of degradation by BOD.

(C) Percentage of degradation by chemical analysis.

(D) Chromatograms or spectra of test chemicals obtained and used for the purpose of analysis.

(v) *Remarks.*

(4) *Interpretation of results.* (i) For the purpose of comparison with reference substances, the biodegradability of the test compound is categorised based on the relative degree of degradability compared to that of aniline.

(ii) If the percentage of degradation of aniline calculated from the oxygen consumption does not exceed 40 percent after 7 days or 65 percent after 14 days, the test is regarded as invalid.

(iii) If the recovery rate of Sb is found to be in the order of 10 percent or less, the test is also regarded as invalid.

See also Section 1.

(d) *Literature references.* For additional background information on this test guideline the following references should be consulted:

(1) *Biodegradability and Bioaccumulation Test of Chemical Substances* (C-5/96/JAP, 1978).

(2) The Chemical Substances Control Law in Japan, Chemical Products Safety Division, Basic Industries Bureau, MITI, (C-2/78/JAP, 1978).

(3) *The Biodegradability and Bioaccumulation of New and Existing Chemical Substances,* 5:8 (C-3/78/JAP, 1978).

[50 FR 39488, Sept. 27, 1985]

### § 796.3240 Ready biodegradability: Modified OECD screening test.

(a) *Introductory information*—(1) *Prerequisites*. (i) Water solubility.

(ii) The organic carbon content of the test material must be established.

(2) *Guidance information*. (i) Information on the relative proportions of the major components of the test material will be useful in interpreting the results obtained, particularly in those cases where the result lies close to the "pass level."

(ii) Information on the toxicity of the chemical may be useful to the interpretation of low results and in the selection of appropriate test concentrations.

(3) *Interpretation of results*. Because of the stringency of this test a result of less than 70 per cent loss of DOC—dissolved organic carbon—(within 28 days) does not necessarily mean that the test compound is not biodegradable under environmental conditions, but indicates that more work will be necessary to establish biodegradability.

(4) *Qualifying statements*. (i) The method is only applicable to those organic test materials which, at the concentration used in the test,

(A) Are soluble in water (at least 5 to 40 mg dissolved organic carbon/liter).

(B) Have negligible vapour pressure.

(C) Are not inhibitory to becteria.

(D) Do not significantly adsorb on glass surfaces.

(ii) This test has been found suitable by the OECD Expert Group Degradation/Accumulation for determining the ready biodegradability of organic chemicals under aerobic conditions. It has been tested in the OECD Laboratory Intercomparison Test Program (1978-1980).

(5) *Recommendations*. (i) Test chemicals giving a result of greater than 70 percent loss of DOC (within 28 days) should be regarded as readily biodegradable. This level is reached within 10 days of biodegradation exceeding 10 percent.

(ii) If the limits of sensitivity of organic carbon analysers are improved, the use of lower test concentrations may be an advantage, particularly for toxic compounds.

(6) *Standard documents*. This Test Guideline constitutes a modification of the OECD Screening Test (OECD) Environment Directorate, Proposed Method for the Determination of the Biodegradability of Surfactants Used in Synthetic Detergents, Paris 1976, and council directive of Nov. 22, 1973, on the approximation of the laws of the member states relating to methods of testing the biodegradability of anionic surfactants (73/405/EEC), Official Journal of the European Communities No. 4 347/53 of Dec. 17, 1973) for the application of the dissolved organic carbon (DOC) analysis.

(b) *Method*—(1) *Introduction, purpose, scope, relevance, application and limits of test*. (i) The purpose of the method is the measurement of the ultimate biodegradability of water soluble, nonvolatile organic compounds in an aerobic, aqueous medium at a starting test concentration corresponding to 5-40 mg DOC/l (In order to avoid inhibitory effects, it is in the investigator's own interest to choose as low a starting concentration as his analytical capability permits.)

(ii) *Definitions and units*. (A) Definition of biodegradability:

$$D_t = \left[1 - \frac{C_t - C_{bt}}{C_o - C_{bo}}\right] \times 100$$

Where:

$D_t$=degradation in percent DOC-removal at time t

$C_o$=starting DOC concentration of the culture medium (mg DOC/l)

$C_t$=DOC concentration of the culture medium at time t (mg DOC/l)

$C_{bo}$=starting DOC concentration of the blank (mg DOC/l)

$C_{bt}$=DOC concentration of the blank at time t (mg DOC/l)

(B) Degradation is stated as the percentage DOC-removal within 28 days with respect to the test material (% DOC-removal).

(iii) *Reference substances*. In some cases when investigating a new substance reference substances may be useful; however, specific reference substances cannot yet be recommended.

In order to check the activity of the inoculum the use of control substances is desirable. Aniline, sodium acetate or sodium benzoate may be used for this purpose. They must exhibit DOC removal >70 percent within 28 days, otherwise the test is regarded as invalid and should be repeated using an inoculum from a different source.

(iv) *Principle of the test method.* (A) A predetermined amount of compound is dissolved in an inorganic medium (mineral nutrient solution, fortified with a trace element and essential vitamin solution), providing a concentration corresponding to 5–40 mg DOC/l. The solution is inoculated with a small number of micro-organisms from a mixed population and aerated at 20–25 °C in the dark or at least in diffuse light only. The degradation is followed by DOC analysis over a 28-day period. The procedure is checked by means of a standard.

(B) A control with inoculation, but without either test material or standard, is run parallelly for the determination of DOC blanks.

(v) *Quality criteria*—(A) *Reproducibility.* The reproducibility of the method is appropriate for a screening test which has solely an acceptance but no rejective function.

(B) *Sensitivity.* The sensitivity of the method is largely determined by the sensitivity limit of the organic carbon analysis, which is 0.5 mg C/liter at present.

(C) *Specificity.* This method is applicable for the biodegradability evaluation of water soluble, non-volatile organic compounds.

(D) *Possibility of standardization.* The test version with specific analyses for anionic and nonionic surfactants is standardized as the "OECD Screening Test."

(E) *Possibility of automation.* (*1*) Parts of the test, e.g., the analysis, can be automated, although hardly the total procedure.

(*2*) The procedure is, however, well suited for being operated with whole series of test materials.

(2) *Description of the test procedure*—(i) *Preparations*—(A) *Reagents*—(*1*) *Deionized water.* (*i*) Deionized or distilled water free of toxic substances (copper in particular), for general use as a solvent. Water which has been deionized by distillation or ion exchange is suitable. Distilled water will never contain more than 10 percent of organic carbon introduced by the test material.

(*ii*) The high purity of this test water is necessary in view of the DOC analyses in the concentration range of 0–40 mg/l. Contamination may result from inherent impurities but also from the ion exchange resins and microbial developments (bacteria, algae under the influence of light, etc.). Only one water charge must be used for each test series, which is to be controlled beforehand by DOC analysis. If necessary, suitable water may be obtained by UV irradiation or other means.

(*2*) *Nutrient solution.* Mix 1 ml each of the following solutions (*i*) to (*vi*) and make up to a volume of 1 liter with water, under paragraph (b)(2)(i)(A)(*1*)(*i*) of this section. (A.R. means analytical reagent).

(*i*)

| | |
|---|---|
| $KH_2PO_4$ | A.R. 8.5 g |
| $K_2HPO_4$ | A.R. 21.75g |
| $Na_2HPO_4 \cdot 2H_2O$ | A.R. 33.4 g |
| $NH_4Cl$ | A.R. 20.0 g |

In 1000 ml of water under paragraph (b)(2)(i)(A)(*1*)(*i*) of this section the pH value should be 7.2.

(*ii*) 22.5 g of $MgSO_4 \cdot 7H_2O$ A.R. dissolved in 1000 ml of water.

(*iii*) 27.5 g of $CaCl_2$ A.R. dissolved in 1000 ml of water.

(*iv*) 0.25 g of $FeCl_3 \cdot 6H_2O$ A.R. dissolved in 1000 ml of water.

This solution is prepared freshly immediately before use.

(*v*) Trace element solution:

| | |
|---|---|
| $MnSO_4 \cdot 4H_2O$ | 39.9 mg (30.23 mg $MnSO_4 \cdot H_2O$) |
| $H_3BO_3$ | 57.2 mg |
| $ZnSO_4 \cdot 7H_2O$ | 42.8 mg |
| $(NH_4)_6Mo_7O_{24}$ | 34.7 mg (36.85 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$) |

Fe—chelate ($FeCl_3$, EDTA), 100 mg; water under paragraph (b)(2)(i)(A)(*1*)(*i*) of this section, 1000 ml.

Sterilization of the trace element stock solution at 120 °C (393 K), 2 atm., 20 min.

(*vi*) Vitamin solution:

| | |
|---|---|
| Biotin | 0.2 mg. |
| Nicotinic acid | 2.0 mg. |
| Thiamine | 1.0 mg. |
| p-Aminobenzoic acid | 1.0 mg. |
| Pantothenic acid | 1.0 mg. |
| Pyridoxamine | 5.0 mg. |
| Cyanocobalamine | 2.0 mg. |
| Folic acid | 5.0 mg. |
| Water (above) | 100 ml. |

The solution is filtered sterile (0.2 μm). Instead of solution (*vi*) under paragraph (b)(2)(i)(A)(*2*) of this section, 15 mg of yeast extract may be used per 100 ml of water under paragraph (b)(2)(i)(A)(*1*)(*i*) of this section.

(*3*) *Control substances.* See Reference substances. Aniline must be freshly distilled.

(B) *Materials.* (*1*) Shaking machine accommodating 2 liters. Erlenmeyer flasks either with automatic temperature control or used in a constant temperature room at 20-25 °C (293-298 K).

(*2*) Narrow neck, 2 liter Erlenmeyer flasks (creased fluted flasks are recommended). The flasks must be carefully cleaned with, e.g., alcoholic hydrochloric acid, before use, rinsed and dried in order to avoid contamination with residues from previous tests. The flasks must also be cleaned before the first use since they may be contaminated.

(*3*) Membrane filtration apparatus.

(*4*) Membrane filters, 0.2 μm.

(*5*) Carbon analyzer.

(C) *Inoculation.* Either of the following three alternatives under paragraphs (b)(2)(i)(C) (*1*), (*2*), and (*3*) of this section may be used as inoculum or a composite sample thereof under paragraphs (b)(2)(i)(C)(*4*) of this section.

(*1*) *Inoculum from secondary effluent.* The inoculum is gained preferably from a secondary effluent of good quality collected from a treatment plant dealing with a predominantly domestic sewage. The effluent must be kept under aerobic conditions in the period between sampling and use. To prepare the inoculum the sample is filtered through a coarse filter, the first 200 ml being discarded. The filtrate is kept aerobic until used. The inoculum must be used on the day of collection.

(*2*) *Inoculum from soil.* (*i*) 100 g of soil (fertile, not sterile) are suspended in 1000 ml of chlorine-free drinking water (soils with an extremely large content of clay, sand or organic carbon are unsuitable). After stirring the suspension is allowed to settle for 30 minutes.

(*ii*) The supernatant is filtered through coarse filter paper, the first 200 ml being discarded. The filtrate is aerated immediately and continuously until use. The inoculum must be used on the day of collection.

(*3*) *Inoculum from a surface water.* (*i*) An inoculum is drawn from a suitable surface water.

(*ii*) The sample is filtered through a coarse paper, the first 200 ml being discarded. The filtrate is kept aerobic until used. The inoculum must be used on the day of collection.

(*4*) *Composite inoculum.* Equal volumes of the 3 inoculum samples are combined, mixed well, and the final inoculum drawn from this mixture. The suitability of the inoculum is checked by means of a control substance.

(ii) *Procedure.* (A) The test materials are evaluated simultaneously in duplicate together with the biodegradability standard and a control test with inoculation but without either test or standard material for the determination of DOC blanks.

(B) The control material must attain >70 percent DOC removal within 28 days at a starting concentration corresponding to 20 mg DOC/l. If <70 percent DOC removal is not achieved the whole series must be discarded. (This limit is based on present experience with the 19-day version of the test. A revision of this limit or even of the standard might have to be considered after the accumulation of experience with the new 28-day version of the test.)

(C) A stock solution of the test material in water under paragraph (b)(2)(i)(A)(*1*)(*i*) of this section is prepared. Enough stock solution is added to the nutrient solution (above) to achieve a carbon concentration of 5-40 mg DOC/l. The starting concentration of the control substance is, however, 20 mg DOC/l.

(D) Two reaction vessels are each filled with 900 ml of the nutrient solution and inoculated with 0.5 ml/l of the inoculum. The opening of the vessel is covered (e.g., aluminum foil) in such a way that the exchange of air between the flask and the surrounding atmosphere is not unduly impeded. (Cotton wool is unsuited because of the DOC analysis.) The vessels are then inserted in the shaking machine. The temperature of 20–25 °C (293–298 K) must be maintained unchanged during the test, and the vessels should be shielded from light. The air should be free of pollutants and toxic materials (chlorinated solvents, etc.)

(E) In the course of the biodegradation test the DOC concentrations are determined in duplicate at the beginning (day 0), and on the 27th and 28th day. Three additional analyses must be performed at regular time intervals (7th, 14th, and 21st day)

(F) The analyses are registered on the form sheet (below) and evaluated.

(G) Only the necessary volumes of culture medium should be drawn for each determination; however, they must be large enough for the membrane filtration or centrifugation preceding the carbon determination. The latter requires differing volumes for the different instruments. Evaporation losses of the culture medium are to be made up by adding water under paragraph (b)(2)(i)(A)(*1*)(*i*) of this section in the required amounts. The culture medium is to be mixed well before withdrawing a sample. Material adhering to the wall of the vessel must be dissolved or suspended before sampling. The membrane filtration or centrifugation must be done immediately. The filtered or centrifuged samples must be analyzed on the same day, otherwise they must be preserved with 0.05 ml of the $HgCl_2$ solution for each 10 ml of nutrient medium or by storing at 2–4 °C. The biodegradability test is valid provided the standard exhibits a degradation rate within the specified range. The test can be finished before the 28th day if complete mineralization has been accomplished. Where degradation has obviously started on day 28 but did not reach a plateau on day 28, it is considered good practice to extend the experiments for one or two weeks longer.

(H) All steps require great care and cleanliness of the vessels, pipettes, etc. but not sterility.

(iii) *Analytical means*—(A) *Membrane filter.* (*1*) 0.2 μm, 25 mm diameter. Preparation of the filters: membrane filters are impregnated with surfactants for hydrophilization. Thus each filter contains up to several mg of soluble carbon which would interfere in the biodegradability determinations. Therefore, the filters are purified from surfactants and other soluble organic interferences by boiling them in deionized water for three periods each of one hour. These filters may be stored in water (above) for at least one week.

(*2*) Other membrane filters are suitable if it is assured that they neither release carbon nor adsorb the compound in the filtration step.

(*3*) If the samples are centrifuged, this must be done at 40,000 $msec^{-2}$ (4000 g) for 15 minutes, preferably in a refrigerated centrifuge, in any case <-40 °C.

(*4*) (Remark: the differentiation TOC: DOC by centrifugation at very low concentrations does not seem to work well since either not all bacteria are removed or carbon as part of the bacterial plasma is redissolved. At higher test concentrations (>10 mg C/l) and the same small inoculation the centrifugation error seems to be comparatively small.)

(B) *The DOC measurement.* (*1*) The sample withdrawn from the culture medium (about 30 ml) is centrifuged or membrane filtered immediately in the filtration apparatus using the membrane filters prepared as stated above. The first 20 ml of the filtrate are discarded.

(*2*) The DOC concentration is determined twice in the remaining filtrate (about 10 ml) by means of the TOC/DOC instrument. If the filtrate cannot be analyzed on the same day it must be preserved as stated above. The DOC measurements (mgC/l) obtained are registered on the attached data sheet and the DOC concentrations of the culture medium and of the blanks calculated for each sampling time.

(c) *Data and reporting*—(1) *Treatment of results.* (i) The degradation at time t is calculated from the determinations of the DOC concentrations at the beginning ($C_0$) and at time t ($C_t$) according to—

$$D_t = \left[1 - \frac{C_t - C_{blt}}{C_0 - C_{bl0}}\right] \times 100$$

where:

$D_t$ = degradation in percent at time t

$C_0$ = measured starting DOC concentration of the inoculated culture medium (mg DOC/l)

$C_t$ = DOC concentration of the culture medium at time t (mg DOC/l)

$C_{bl0}$ = starting DOC blank of the mineral nutrient solution with inoculation, but without test material (mg DOC/l)

$C_{blt}$ = DOC blank of the mineral nutrient solution with inoculation, but without test material at time t (mg DOC/l)

(ii) The degradation rates are calculated to the nearest 0.1 percent. The means of the $D_t$ values are calculated and reported to the nearest full percent. Results ending in 0.5 are rounded up to the nearest whole number. The course of the degradation test is presented graphically in a diagram.

(2) *Test report.* The results may be reported on the following data sheet:

## SAMPLE FORM SHEET FOR THE MODIFIED OECD SCREENING TEST

Exp. no.: ______________________

Date of start of test: ______________________

Test : Standard material: ______________________

Theoretical test conc.: ______________________ mg DOC/l

Inoculum: ______________________

Carbon analyser: ______________________

PART I: CONTROLS:

| | | |
|---|---|---|
| Stock solution of the test material (1000 mg/l, dilution .../1000 ml of nutrient solution) | TOC[1] | DOC[2] |
| | mg/l | |
| | | |

PART II: CARBON DETERMINATIONS:

| Culture medium | Flask No. | Analyses | Theor. conc. mg/l | DOC - concentrations after x days mg/l | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | 0 ($C_0$) | ~7 | ~14 | ~21 | ~27 | 28 |
| Mineral nutrient solution with test material and with inoculum | 1 | $a_1$ | | | | | | | |
| | | $a_2$ | | | | | | | |
| | | $m_1 = \frac{a_1 + a_2}{2}$ ($C_t$) | | | | | | | |
| | 2 | $b_1$ | | | | | | | |
| | | $b_2$ | | | | | | | |
| | | $m_2 = \frac{b_1 + b_2}{2}$ ($C_t$) | | | | | | | |
| Mineral nutrient solution without test material but with inoculum | Blank | $c_1$ | | | | | | | |
| | | $c_2$ | | | | | | | |
| | | $m_3 = \frac{c_1 + c_2}{2}$ ($C_{b1}$) | | | | | | | |

PART III: EVALUATION OF RAW DATA:

| | Flask No. | | % DOC removal after x days | | | | |
|---|---|---|---|---|---|---|---|
| | | | 7 | 14 | 21 | 27 | 28 |
| DOC - concentrations minus blanks | 1 | $D_1 = \left[1 - \frac{m_{1_t} - m_{3_t}}{m_1 - m_3}\right] . 100$ | | | | | |
| | 2 | $D_2 = \left[1 - \frac{m_{2_t} - m_{3_t}}{m_2 - m_3}\right] . 100$ | | | | | |
| | mean | $D = \frac{D_1 + D_2}{2}$ for day x | | | | | |

---

[1] Disagreement between DOC and TOC values points towards insufficient solubility of the test material.

[2] All DOC values determined after membrane filtration.

(3) *Interpretation of results.* (i) The results of the degradation test are valid if the condition is met that in the same test series the control yields 70 percent DOC-removal.

(ii) Because of the stringency of this test a result inferior to the recommended pass level (70 percent loss of DOC) does not necessarily mean that the test compound is not biodegradable under environmental conditions, but indicates that more work will be necessary to establish this.

[50 FR 39496, Sept. 27, 1985]

### § 796.3260 Ready biodegradability: Modified Sturm test.

(a) *Introductory information*—(1) *Prerequisites.* The total organic carbon content of the test material should be calculated or, if this is not possible, analyzed to enable the theoretical yield of $CO_2$ to be calculated.

(2) *Guidance information.* (i) Information on the relative proportions of the major components of the test material will be useful in interpreting the results obtained, particularly in those cases where the result lies close to the "pass level."

(ii) Information on the toxicity of the chemical may be useful in the interpretation of low results and in the selection of appropriate test concentrations.

(3) *Interpretation of results.* Because of the stringency of this test, a result of less than 60 percent yield of $CO_2$ (within 28 days) does not necessarily mean that the test compound is not biodegradable under environmental conditions, but indicates that more work will be necessary to establish biodegradability.

(4) *Qualifying statements.* (i) The method is only applicable to those organic test materials which, at the concentration used in the test:

(A) Have negligible vapour pressure.

(B) Are not inhibitory to bacteria.

(C) Do not significantly adsorb to glass surfaces.

(ii) This test has been found suitable by the OECD Expert Group Degradation/Accumulation for determining the ready biodegradability of organic chemicals under aerobic conditions. It has been tested in the OECD Laboratory Intercomparison Test Programme (1978-1980).

(iii) Evolution of a significant amount of $CO_2$ from the blank flask during the test would indicate contamination of the medium, glassware or air supply. A total $CO_2$ evolution in the blank at the end of the test exceeding 50 mg $CO_2$ per 3 liters medium should be considered as invalidating the test.

(5) *Recommendations.* (i) Test chemicals giving a result of greater than 60 percent yield of $CO_2$ (within 28 days) should be regarded as readily biodegradable. This level must be reached within 10 days of biodegradation exceeding 10 percent.

(ii) If the test material is not soluble at the test concentration, special measures, such as the use of ultrasound dispersion, may have to be employed to achieve a good dispersion of the test material.

(6) *Standard documents.* This test guideline is based on biodegradability test methods described in paragraphs (d) (1) and (2) of this section. Various improvements have been made in the test protocol, the latest ones being the object of the paper cited in paragraph (d)(3) of this section.

(b) *Method*—(1) *Introduction, purpose, scope, relevance, application and limits of test.* (i) (A) This test guideline has been developed for screening readily biodegradable chemicals. When a broader understanding of the biodegradability of a compound is required (particularly for new chemicals never discharged into the environment) environmentally relevant results may be obtained by operating the same test guideline but with inoculum preadapted to the test compound under paragraphs (d) (1), (2), and (3) of this section.

(B) A high biodegradation result in this test provides the evidence that the test compound is highly biodegradable in aerobic systems.

(C) On the contrary, a low biodegradation result may have other causes than poor biodegradability of the test compound. Inhibition effects of the inoculum by the toxicity of the compound at the test concentration is often a cause for low biodegradation. In such cases the result is meaningless and further work is needed to assess the biodegradability of the test compound in systems and at concentrations where inhibition effects are overcome.

(D) An estimate of the expected environmental concentration will help to put toxicity effects into perspective and determine test concentrations.

(E) The proportion of carbon incorporated into cellular material to carbon released as CO$_2$ will vary depending on the organic substrate, on the particular micro-organism(s) carrying out the conversion and on the environmental conditions under which the conversion takes place. In principle, this uncertainty is a drawback in the interpretation of the results from this test.

(F) However, it has been observed that under the conditions of this test with a test compound concentration (sole source of carbon) ranging from 5 to 20 mg/l, a majority of substrate carbon is used to drive catabolic processes and a minimum is used to generate new cells under paragraph (d)(3) of this section. Under these conditions, the measure of CO$_2$ evolution becomes an accurate measure of the rate and extent of catabolism of a given material.

(ii) *Definitions and units.* The amount of CO$_2$ produced by the test compound during the test is measured and expressed as percent of the theoretical CO$_2$ it should have produced (TCO$_2$) calculated from the carbon content of the test compound. Biodegradability is therefore expressed as percentage TCO$_2$.

(iii) *Reference substances.* In some cases when investigating a new substance reference substances may be useful; however, specific reference substances cannot yet be recommended. In order to check the activity of the inoculum, the use of control substances is desirable. Aniline, sodium acetate or sodium benzoate may be used for this purpose. They must give a yield >60 percent CO$_2$ within 28 days, otherwise the test is regarded as invalid and should be repeated using an inoculum from a different source.

(iv) *Principle of the test method.* (A) A chemically defined liquid medium, essentially free of other organic carbon sources, is spiked with the test material and inoculated with sewage micro-organisms. The CO$_2$ released is trapped as BaCO$_3$.

(B) After reference to suitable blank controls, the total amount of CO$_2$ produced by the test compound is determined for the test period and calculated as the percentage of total CO$_2$ that the test material could have theoretically produced based on carbon composition.

(v) *Quality criteria*—(A) *Reproducibility.* In the absence of toxicity effects of the test compound on the inoculum, the reproducibility is around ±5 percent, relative. When toxicity effects start occurring, the reproducibility becomes poor.

(B) *Sensitivity.* The endogenous CO$_2$ production of the inoculum as measured in the blank flask (no test material) is the main reason why the test cannot use test compound concentrations lower than 5 mg/l. (When the test is adapted to handle $^{14}$C labelled test compounds, test compound concentrations can be very much lower.)

(C) *Specificity.* (*1*) This test is adequate for soluble and insoluble organic materials. The test material must not be volatile. The purity of the test material should be high since organic impurities will complicate the interpretation of the CO$_2$ production data.

(*2*) Test results are meaningful only if, at the test concentration, the material has no toxic effect on the inoculum.

(D) *Possibility of standardization.* This possibility exists. The major difficulty is to standardise the inoculum in such a way that interlaboratory reproducibility is ensured. The selection of test organisms and their handling in the laboratory as described below (Test organisms) is one way to ensure "healthy" inoculum with a mutiplicity of micro-organism species. Procedures using preadaptation of the inoculum to the test compound usually reach higher reproducibility.

(E) *Possibility of automation.* None at present.

(2) *Description of the test procedure*—(i) *Preparations*—(A) *Apparatus equipment*—(*1*) *CO$_2$ scrubbing apparatus:* For a series of 12 carboys (3 test materials):

(*i*) Four 1-liter plastic bottles, filled with 700 ml 10 N NaOH.

(*ii*) One 1-liter Erlenmeyer flask containing 700 ml 0.025 N $Ba(OH)_2$ solution.

(*iii*) One empty 1-liter Erlenmeyer to prevent liquid carry-over.

These bottles are connected in series, using Tygon tubing, to a pressurized air source, and air is sparged through the scrubbing solutions at a constant rate. For each additional set of carboys, add 1 additional 1-liter plastic bottle filled with 700 ml 10 N NaOH.

(*2*) *$CO_2$ production apparatus.* (*i*) Four 5-liter disposable amber carboys for each test material.

(*ii*) Stoppers, flexible tubing, plastic tubing.

(*iii*) "French squares" (i.e. 100 ml barium hydroxide absorber bottles) or similar containers.

(*3*) *Analytical equipment.* (*i*) (Carbon analyzer: optional if sample is well characterized.)

(*ii*) Analytical balance.

(*iii*) 100 ml-buret.

(B) *Chemicals/reagents/materials*—(*1*) *Stock solutions for test medium*—(*i*) *Ferric chloride solution:* dissolve 0.25 g $FeCl_3 \cdot 6H_2O$ in 1 liter distilled water.

(*ii*) *Magnesium sulfate solution:* dissolve 22.5 g $MgSO_4 \cdot 7H_2O$ in 1 liter distilled water.

(*iii*) *Calcium chloride solution:* dissolve 27.5 g anhydrous $CaCl_2$ in 1 liter distilled water.

(*iv*) *Phosphate buffer solution:* dissolve 8.5 g $KH_2PO_4$, 21.75 g $K_2HPO_4$, 33.4 g $Na_2HPO_4 \cdot 7H_2O$ and 1.7 g $NH_4Cl$ in 1 liter distilled water.

(*v*) *Ammonium sulfate solution:* dissolve 40 g $(NH_4)_2SO_4$ in 1 liter distilled water.

(*2*) *Test medium.* The test medium will contain per liter of high quality water (High quality water, free of toxic substances (copper, in particular), with low carbon content (<2.0 mg/liter TOC) and with a resistivity >18 megaohms.cm. (Distilled water must never contain more than 10 percent of organic carbon introduced by the test material.)) the following reagents:

(*i*) 1 ml of magnesium sulfate solution under paragraph (b)(2)(i)(B)(*1*)(*ii*) of this section.

(*ii*) 1 ml of calcium chloride solution under paragraph (b)(2)(i)(B)(*1*)(*iii*) of this section.

(*iii*) 2 ml of phosphate buffer solution under paragraph (b)(2)(i)(B)(*1*)(*iv*) of this section.

(*iv*) 4 ml of ferric chloride solution under paragraph (b)(2)(i)(B)(*1*)(*i*) of this section.

(*v*) 1 ml of ammonium sulfate solution under paragraph (b)(2)(i)(B)(*1*)(*v*) of this section.

(*3*) *Barium hydroxide, 0.025 N.* Dissolve 4.0 g $Ba(OH)_2 \cdot 8H_2O$ per liter high quality water. Filter through filter paper and seal the clear solution to prevent absorption of $CO_2$ from the air. It is wise to prepare more than 5 liters at a time when running series.

(*4*) *Test materials.* (*i*) Basic physical-chemical data regarding water solubility, impurities solvent and percentage active must be specified by the sample submitter.

(*ii*) An initial stock solution is prepared from the test material by weighing out a homogeneous aliquot of the sample. The sample is dissolved in high quality water to give a solution of test material of 1000 mg/l. Stock solutions are made up on the basis of percentage of active compound in the test material. If the percentage of active compound is unknown, stock solutions are made up to concentration of 1000 mg/l on a weight basis. To obtain a homogeneous sample, it may be necessary to mix well, at the same time avoiding foaming which will tend to concentrate the active ingredient disproportionally. For solid samples, it may be necessary to melt and mix the entire contents of the sample bottle before taking the aliquot. This portion of the procedure is extremely important since the calculations of percentage of biodegradation depend directly on having added the correct amount of carbon to the test system.

(*iii*) The pH of the stock solution need not be adjusted unless it falls outside the range of 3-10, since the phosphate buffer in the test medium will control it. If the pH lies outside this range, adjust an aliquot of the stock solution to pH 7.0 (±1.0) with 1 N HCl or NaOH, making sure that the solution is being vigorously mixed during the addition of acid or base.

(*iv*) To confirm the nominal concentration of organic carbon of the test compound, the stock solution (or the neutralized aliquot) may be analyzed for total organic carbon (TOC). A TOC analysis is also required for the control stock solution.

(*v*) If a test material is insoluble in water, add the appropriate amount of test material directly to the carboy on a weight or volume basis. Total organic carbon analysis cannot be performed on insoluble test materials.

(*5*) *Test organisms.* (*i*) The source of test organisms is activated sludge freshly sampled from a well-operated municipal sewage treatment plant. This sewage treatment should receive no or minimal effluents from industry.

(*ii*) Upon arrival at the laboratory, the activated sludge is aerated for 4 hours. 500 ml of the mixed liquor is sampled and homogenized for 2 minutes at medium speed in a Waring blender. It is then settled for ½ hour.

(*iii*) If the supernatant still contains high levels of sludge solids at the end of 30 minutes, it may be settled for an additional 30–60 minutes or adapted to laboratory conditions to obtain better settleability.

(*iv*) The supernatant is decanted to provide sufficient volume for a 1 percent inoculum for each $CO_2$ test flask. Avoid carryover of sludge solids which would interfere with the measurement of $CO_2$ production.

(*v*) Although optional, it is useful to perform viable counts on the supernatant fraction to determine microbial numbers. This inoculum should normally contain $10^6$ to $20 \times 10^6$ colony forming units per ml. It should be used on the day it is prepared. The $CO_2$ production test then proceeds as follows.

(ii) *Test conditions.* (A) (*1*) Since a 1 percent inoculum is used in the $CO_2$ test, it is necessary to make dilutions in the $CO_2$ test medium.

(*2*) This is most easily achieved as follows:

(*i*) To each of the 5-liter test carboys, add 2470 ml of high quality water.

(*ii*) To each of the 5-liter test carboys, add 3 ml each of the ammonium sulfate, magnesium sulfate, and calcium chloride stock solutions; add 6 ml of the phosphate buffer stock solution and 12 ml of the ferric chloride solution.

(*iii*) To each of the 5-liter test carboys, add 30 ml of the activated sludge inoculum.

(*iv*) These additions now total 2527 ml in each carboy.

(B) This mixture is aerated with $CO_2$-free air for 24 hours, to purge the system of carbon dioxide (see $CO_2$ scrubbing apparatus, above).

(C) After the aeration period, three $CO_2$ absorber bottles are filled with 100 ml 0.025 N $Ba(OH)_2$ and connected in series to the exit air line of each test carboy.

(D) Test material is added to two of the four carboys to begin the testing period. Each material is tested at two concentrations: 10 and 20 mg/l. The amount of test material stock solution required in the carboy is calculated as follows:

$$\text{ml of stock solution per carboy is: } \frac{B \times C}{A}$$

where:

B is test compound concentration in test carboy (mg/l),

A is test compound concentration in stock solution (mg/l),

C is final volume of test medium in test carboy (ml).

(E) Sufficient stock solution to reach the desired test concentration, as calculated above, plus enough distilled water to make 473 ml (stock solution+high quality water) are added to the appropriate carboys. To the third carboy, used as blank control and containing no test material, 473 ml of high quality water are added. The final volume of each carboy is now 3000 ml.

(F) A control substance at a concentration of 20 mg/l is added to the last of the four carboys (see paragraph (d) of this section on reference substances.

(iii) *Performance of test.* (A)(*1*) The test is started by bubbling $CO_2$-free air through the solution at a rate of 50-100 ml/min per carboy (approximately 1-2 bubbles/second). The $CO_2$ produced in each carboy reacts with the barium hydroxide and is precipitated out as barium carbonate; the amount of $CO_2$ produced is determined by titrating the remaining $Ba(OH)_2$ with 0.05 N standardized HCl (see below). Periodically (every 2 or 3 days), the $CO_2$ absorber nearest the carboy is removed for titration. The remaining two absorbers are each moved one place closer to the carboy, and a new absorber filled with 100 ml of fresh 0.025 N $Ba(OH)_2$ is placed at the far end of the series. Titrations are made as needed (before any $BaCO_3$ precipitate is evident in the second trap), approximately every other day for the first 10 days, and then every fifth day until the 28th day.

(*2*) For water-insoluble test materials, incorporated dry into the $CO_2$ test carboy, agitation can be done with a magnetic stirrer. For foaming chemicals, $CO_2$-free air bubbling can be replaced by overhead aeration and magnetic stirring.

(B) On the 26th day, the pH of the carboy contents is measured again, and then 1 ml of concentrated HCl is added to each of the test carboys to drive off inorganic carbonate. The carboys are aerated overnight, and samples are removed from each carboy for dissolved organic carbon (DOC) analysis. The final titration is made on day 28.

(C) Titrations of the 100-ml $Ba(OH)_2$ solution are made after removing the bottles closest to the carboys. The $Ba(OH)_2$ is titrated with 0.05 N HCl, using phenophthalein as an indicator.

(D) The test is run at room temperature and temperature is recorded during the test period.

(c) *Data and reporting*—(1) *Treatment of results*—(i) *$TCO_2$*. (A) The theoretical amount of $CO_2$ that can be generated by a test material, or $TCO_2$, is calculated as follows:

$$TCO_2 = \text{mg } CO_2/\text{mg test material} = \frac{\text{N° of carbons in test material} \times \text{mol. wt. of } CO_2}{\text{mol. wt. of active test material}}$$

(B) For instance, dextrose ($C_6H_{12}O_6$) contains 6 carbon, therefore $TCO_2$ of dextrose=

$$\frac{6 \times 44}{180} = 1.467 \text{ mg } CO_2/\text{mg dextrose}$$

(C) For mixtures, the $TCO_2$ of the total active material is a weighted average of the $TCO_2$'s of the individual components.

(ii) *Amount of $CO^2$ produced.* (A) The first step in calculating the amount of $CO_2$ produced is to correct the test material carboys for endogenous $CO_2$ production. The control carboy serves as a "seed blank" to correct for $CO_2$ which may be produced through endogenous respiration of the bacteria. The amount of $CO_2$ produced by a test material is determined by the difference (in ml of titrant) between the experimental and blank $Ba(OH)_2$ traps.

For example:

| | |
|---|---|
| Blank | 48.0 ml HCl titrated |
| Experimental | 45.0 ml HCl titrated |
| Test material | 3.0 ml HCl titrated |

(B) The next step is to convert ml HCl titrated into mg of $CO_2$ produced. When $CO_2$ enters the absorber bottle, it reacts in the following manner:

$$Ba(OH)_2 + CO_2 \rightarrow BaCO_3 \downarrow + H_2O$$

(C) The $BaCO_3$ formed is insoluble and precipitates. The amount of $Ba(OH)_2$ remaining in solution is determined by titration of the 100 ml with HCl according to the following equation:

$$Ba(OH)_2+2HCl \rightarrow BaCl_2+2H_2O$$

(D) From the above two equations, it can be seen that 1 mmol of $CO_2$ is produced for every 2 mmol of HCl titrated. This means that the number of mmol of $CO_2$ produced:

$$\text{mmol } CO_2 = \frac{\text{mmol HCl}}{2}$$

(E) The normality of HCl used is 0.05 N. Substituting for mmol gives:

$$\text{mmol } CO_2 = \frac{(0.05\text{ N})\times(\text{ml of HCl}}{2}$$

(F) To convert to mg $CO_2$, the value must be multiplied by the molecular weight of $CO_2$ which is 44:

$$\text{mg } CO_2 = \frac{((0.05)\times\text{ml titrated}}{2} \times 44 = 1.1\times\text{ml of HCl titrated})$$

(G) Thus, to convert ml of HCl to mg $CO^2$, the former is multiplied by 1.1.

(iii) *Percentage of theoretical $CO_2$.* (A) The percentage of theoretical $CO_2$ produced is calculated from the following formula:

$$\%TCO_2 = \frac{\text{mg } CO_2 \text{ produced}}{(\text{mg test material added in test})\ (\text{mg } CO_2/\text{mg test material})} \times 100$$

$$= \frac{\text{mg } CO_2 \text{ produced}}{(\text{mg test material added in test})\ (TCO_2)} \times 100$$

EXAMPLE

| | |
|---|---|
| Blank | 48.0 ml 0.05 N HCl titrated |
| Dextrose | 45.0 ml 0.05 N HCl titrated |
| Difference | 3.0 ml 0.05 N HCl titrated |

(B) When multiplied by 1.1, 3.0 is equivalent to 3.3 mg $CO_2$ produced.

(C) Since dextrose has a $TCO_2$ of 1.47 mg $CO_2$/mg dextrose, and since 60 mg of dextrose were placed in the test (i.e. 3 litres×20 mg/l), the percentage of theoretical $CO_2$ that was degraded on the particular day was:

$$\%TCO_2 = \frac{3.3\text{ mg } CO_2 \text{ produced}}{(60\text{ mg dextrose})\ (1.47\text{ mg } CO_2/\text{mg dextrose})} \times 100 = 3.74$$

(2) *Test report.* (i) The test material should be well identified (source, physical-chemical data, organic carbon content, purity, etc.).

(ii) The test concentration at start should be reported with end results.

(iii) Indicate date and location where test organisms were sampled.

(iv) COD and TOC analyses on the stock solution of the test compound should be reported.

(v) Temperature range recorded during the test period must be noted.

(vi) If measured as suggested above (Test organisms), report number of micro-organisms per ml (colony forming units—CFU/ml).

(vii) For each titration, calculate percentage $TCO_2$ evolved. Plot cumulative percentage $TCO_2$ versus time until the end of the test. Not only the end result is important, but also the length of the lag phase and the slope (rate).

(viii) If the curve reaches a plateau before 28 days, the test can be ended and the plateau value considered as final.

(ix) If the curve shows that biodegradation started before day 28 but the plateau is not reached at day 28, then the test should be prolonged until the plateau is reached.

(x) If a more rigorous mathematical treatment of the data is desired, the cumulative $CO_2$ versus time data can be fit into a non-linear regression model to generate rate constants for mineralization and a final extent of degradation at infinite time (asymptote) (3).

(3) *Interpretation of results.* See paragraph (a) of this section.

(d) *Literature* references. For additional background information on this test guideline the following references should be consulted:

(1) Thompson, J.E., and Duthie, J.R., "The biodegradability of NTA," *Journal of Water Pollution Control Federation.* 40: 306-319 (1968).

(2) Sturm, R.N., "Biodegradability of Nonionic Surfactants: Screening Test for Predicting Rate and Ultimate Biodegradation," *Journal of American Oil Chemistry Society. 50:* 159-167 (1973).

(3) Larson, R.J., "Estimation of Biodegradation Potential of Xenobiotic Organic Chemicals," *Applied Environmental Microbiology. 38:* 1153-1161 (1979).

(4) American Public Health Association. *Standard Methods for the Examination of Water and Wastewater.* 14th Ed, (American Public Health Association, Inc.: New York, 1975) pp. 544-545.

(5) AFNOR T-90-302 Test, *Methode d'evaluation en milieu aqueux de biodegradabilité des produits organiques* (1977).

(6) ISO TC 147 SC5 WG4, OECD Screening Test modified for the use of dissolved organic carbon analysis (1978).

[50 FR 39499, Sept. 27, 1985, as amended at 53 FR 12526, Apr. 15, 1988]

## § 796.3300 Simulation test—aerobic sewage treatment: Coupled units test.

(a) *Introductory information*—(1) *Prerequisites.* (i) Water solubility.

(ii) The organic carbon content of the test material must be established.

(2) *Guidance information.* (i) Information on the relative proportions of the major components of the test material will be useful in interpreting the results obtained, particularly in those cases where the result lies close to the "pass level".

(ii) Information on the toxicity of the chemical may be useful to the interpretation of low results and in the selection of appropriate test concentrations.

(3) *Qualifying statements.* (i) The method is only applicable to those organic test materials which, at the concentration used in the test:

(A) Are soluble in water to the extent necessary for the preparation of the test solutions.

(B) Have negligible vapor pressure.

(C) Are not inhibitory to bacteria.

(D) Do not significantly adsorb on glass surfaces.

(ii) This test is recommended by the OECD Expert Group on Degradation/Accumulation as a test for the determination of the ultimate biodegradability of test materials under conditions which stimulate treatment in an activated sludge plant.

(4) *Standard documents.* This test guideline constitutes a modification of the OECD Confirmatory Test (OECD

Environmental Directorate, Proposed Method for the Determination of the Biodegradability of Surfactants Used in Synthetic Detergents, Paris 1976, and Council Directive of Nov. 22, 1973, on the Approximation of the Laws of the Member States Relating to Methods of Testing the Biodegradability of Anionic Surfactants [73/405/EEC], Official Journal of the European Communities No. 4 347/53 of Dec. 17, 1973). The modifications are described in the references cited under paragraphs (d) (4) and (5) of this section.

(b) *Method*—(1) *Introduction, purpose, scope, relevance, application and limits of test.* (i) The purpose of the method is the measurement of the ultimate biodegradability of the water-soluble, nonvolatile organic compounds in an activated sludge plant model at a concentration corresponding to > 12 mg DOC/l (Dissolved organic carbon/litre) (or approximately 40 mg COD/l (Chemical oxygen demand/liter)). twenty mg DOC/l seem to be optimal.

(ii) *Definitions and units.* (A) Definition of *biodegradability:*

$$DR = \frac{T-(E-E_o)}{T} \times 100$$

Where:

DR=degradation rate in percent DOC (or COD) removal within the given mean retention time with respect to the test material

T=concentration of the test material in mg DOC/litre (or mg COD/litre)

E=DOC (or COD) concentration in the effluent of the test unit in mg DOC/litre (or mg COD/litre)

$E_o$=DOC (or COD) concentration in the effluent of the blank unit in mg DOC/litre (or mg COD/litre)

(B) *Units.* The degradation is stated as the percentage DOC (or COD) removal within the given retention time with respect to the test material (percentage DOC [or percentage COD] removal).

(iii) *Reference substances.* In some cases when investigating a new substance reference substances may be useful; however specific reference substances cannot yet be recommended.

(iv) *Principle of the test method.* Two OECD Confirmatory Test units, i.e., model activated sludge plants, are operated in parallel whereby the parallelism is enhanced and assured by a transinoculation procedure. The test material is added to the influent (synthetic sewage) of one unit while the other is fed only with the synthetic sewage. The DOC (or COD) concentrations are measured in both effluents. The DOC (or COD) difference of these effluent values is due to non- or only partially degraded test material. The raw data obtained require a statistical procedure, i.e. the tolerance limits of the mean must be calculated in addition to the mean.

(v) *Quality criteria*—(A) *Reproducibility.* The reproducibility of the results obtained by this method is satisfactory.

(B) *Sensitivity.* A starting concentration of the test material corresponding to 12 mg DOC/litre (or approximately 40 mg COD/litre) constitutes the sensitivity limit at a mean retention time of 3 hrs; at a 6 hr mean retention time 9-10 mg DOC/litre are sufficient.

(C) *Specificity.* The method is applicable to the biodegradability evaluation of water-soluble, non-volatile organic compounds.

(D) *Possibility of standardization.* The test version with specific analyses for anionic and nonionic surfactants is standardized as the "OECD Confirmatory Test".

(E) *Possibility of automation.* Parts of the test, e.g., the analysis, can be automated, although not the total procedure.

(2) *Description of the test procedure*—(i) *Preparations*—(A) *Apparatus.* (*1*)(*i*) OECD Confirmatory Test Units: The method employs two small activated sludge plants shown in the following Figure 1, and in greater detail in Figure 2, for each biodegradability determination.

**Figure 1—Schematic of OECD Confirmatory Test Activated Sludge Uni**

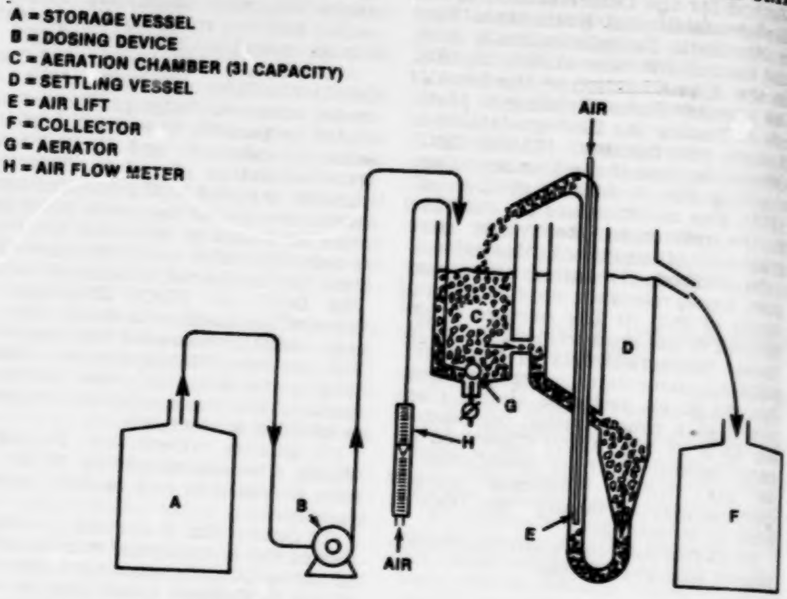
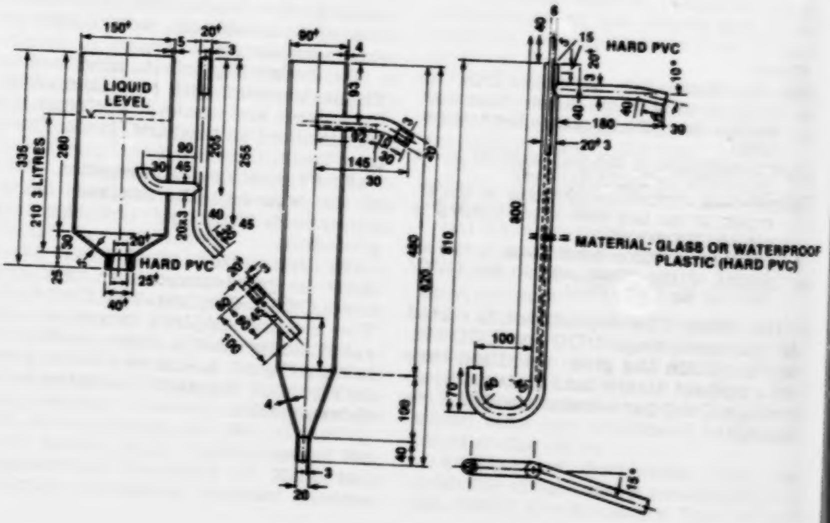

A = STORAGE VESSEL
B = DOSING DEVICE
C = AERATION CHAMBER (3l CAPACITY)
D = SETTLING VESSEL
E = AIR LIFT
F = COLLECTOR
G = AERATOR
H = AIR FLOW METER
AIR
C
D
G
H
A
B
AIR
E
F

**Figure 2—Dimensions of OECD Confirmatory Test Activated Sludge Unit**


150°
5
20°
3
LIQUID LEVEL
335
280
210 3 LITRES
90
30
45
205
255
40
45
20x3
30
20°
HARD PVC
25
25°
40°
90°
4
93
82
10
30
145
30
490
520
810
100
4
100
40
3
20
6
40
15
HARD PVC
20°
10°
40
180
30
20° 3
800
MATERIAL: GLASS OR WATERPROOF PLASTIC (HARD PVC)
100
70
15°

(*ii*) The equipment consists of a storage vessel (A) for synthetic sewage, dosing pump (B), aeration vessel (C), separator (D), air-lift pump (E) to recycle the activated sludge, and vessel (F) for collecting the treated effluent.

(*iii*) Vessels (A) and (F) must be of glass or suitable plastic and hold at least 24 litres. Pump (B) must provide a constant flow of synthetic sewage to the aeration vessel; during normal operation this vessel contains 3 litres of mixed liquor. A sintered aeration cube (G) is suspended in vessel (C) at the apex of the cone. The quantity of air blown through the aerator should be monitored by means of a flowmeter.

(*2*) Carbon analyzer.

(*3*) Membrane filtration apparatus and membrane filters with a pore width of 1.2 μm.

(*4*) Glassware as it is commonly needed for sample preparation.

(*5*) Synthetic sewage: For the test a synthetic sewage is employed. Dissolve in each litre of tap water:

(*i*) 160 mg peptone.

(*ii*) 110 mg meat extract.

(*iii*) 30 mg urea.

(*iv*) 7 mg NaCl.

(*v*) 4 mg $CaCl_2 \cdot 2H_2O$.

(*vi*) 2 mg $MgSO_4 \cdot 7H_2O$.

(B) *Inoculation.* (*1*) A composite inoculum is used.

(*i*) *Inoculum from secondary effluent.* The inoculum should be obtained from a secondary effluent of good quality collected from a treatment plant dealing with predominantly domestic sewage. The effluent must be kept under aerobic conditions in the period between sampling and use. To prepare the inoculum the sample is filtered through a coarse filter, the first 200 ml being discarded. The filtrate is kept aerobic until used. The inoculum must be used on the day of collection.

(*ii*) *Inoculum from soil.* 100 g of garden soil (fertile, not sterile) are suspended in 1000 ml of chlorine-free drinking water. (Soils with an extremely large fraction of clay, sand or humus are unsuitable.) After stirring, the suspension is allowed to settle for 30 minutes. The supernatant is filtered through a coarse filter paper, the first 200 ml being discarded. The filtrate is aerated immediately and until use. The inoculum must be used on the day of collection.

(*iii*) *Inoculum from a surface water.* A further partial inoculum is drawn from a mesosaprobic surface water. The sample is filtered through a coarse paper, the first 200 ml being discarded. The filtrate is kept aerobic until used. The inoculum must be used on the day of collection.

(*2*) Equal volumes of the 3 partial inoculum samples are united, mixed well, and the final inoculum drawn from this mixture.

(ii) *Procedure.* (A) Initially, fill aeration vessels (C) and separator (D) of both units needed for one test with synthetic sewage. (It is strongly recommended that the synthetic sewage be prepared first in one batch. Then the storage vessels (A) of both units needed for one test are filled with the same synthetic sewage.) The height of separator (D) is so fixed that the volume contained in aeration vessel (C) is 3 litres. Inoculation is made by introducting 3 ml of the composite inoculum, above. The aerator, air lift (E) and dosing device (B) are then set in operation. The synthetic sewage must pass through aeration vessel (C) at the rate of one litre per hour; this gives a mean retention time of 3 hours; a rate of one half litre per hour results in a mean retention time of 6 hrs.

(B) The rate of aeration should be regulated so that the contents of vessel (C) are kept constantly in suspension while the dissolved oxygen content is at least 2 mg/l. Foaming must be prevented by appropriate means. Antifoaming agents which inhibit the activated sludge must not be used. Airlift pump (E) is set so that the activated sludge from the separator is continually and regularly recycled to aeration vessel (C). Sludge which has accumulated around the top of the aeration vessel (C), in the base of the settling vessel (D), or in the circulation circuit must be returned to the circulation at least once each day by brushing or some other appropriate means. When sludge fails to settle, its density may be increased by addition of 2-ml portions of a 5 percent solution of ferric chloride, repeated as necessary.

(C) The effluent from separator (D) is accumulated in vessel (F) for 24 hours, following which a sample is taken after thorough mixing. Vessel (F) must be carefully cleaned.

(D) In order to control the efficiency of the process the chemical oxygen demand (COD) or the dissolved organic carbon (DOC) of the filtrate of the effluent (especially of the blank unit) accumulated in vessel (F), is measured at least twice weekly, as well as that of the filtered synthetic sewage in vessel (A).

(E) The reduction in COD or DOC should level off when a roughly regular daily degradation is obtained, i.e. at the end of the running-in period.

(F) The dry matter content of the activated sludge in the aeration tank should be determined twice a week (in g/l). If it is more than 2.5 g/l, the excess activated sludge must be discarded.

(G) The test is performed at room temperature; this should be steady and should be kept between 18 °C and 25 °C.

(H) Two OECD Confirmatory Test units are run parallel in the fashion described above. Sufficient stock solution (approximately 1 percent) of the test material is added to the influent of the test unit that the desired concentration of test material (= approximately 10-20 mg DOC/l or 40 mg COD/l) in the synthetic sewage is obtained. The blank unit is fed only with synthetic sewage.

(I) The coupling of the units is achieved by interchanging 1.5 litres—including sludge—from the activated sludge vessels between the two units once a day. In case of strongly adsorbing test materials 1.5 litres are drawn from the settling vessels and interchanged.

(J) The DOC (or COD) concentrations of the effluents of the test (E) and blank ($E_o$) units are determined daily after membrane filtration (pore size 1.2 μm).

(K) The working-in time should not exceed 6 weeks and the evaluation period should not be shorter than 3 weeks, i.e., about 20 determinations should be available for calculation of the final results.

(iii) *Analytical means.* (A) 250 m
fluent samples are drawn and imm
ately membrane filtered (1.2 μm).
first half of the filtrate is discar
The DOC (or COD) concentratio
determined in the remainder. If
analysis cannot be performed on
same day, the sample is stored in a
frigerator, but only after membr
filtration.

(B) The DOC concentrations are
termined in duplicate with the carb
analyzer and the COD values acco
ing to APHA under paragraph (d)
of this section. The data are record
on the following Figure 3 form she

FIGURE 3—FORM SHEET FOR THE OECD C
FIRMATORY TEST MODIFIED FOR THE AP
CATION OF UNSPECIFIC ANALYSES (COUP
UNITS TEST.)

Exp. No .................... ______
Date of start of test .................... ______
Test material .................... ______
Theoretical test concentration:
  mg active substance/litre (or mg/l of product). ______
  mg DOC/litre .................... ______
  mg COD/litre .................... ______
Inoculum .................... ______
Analysis or analytical instrument. ______
Mean retention time: (hrs) .................... ______
Volume being interchanged ($v_i$): (l/d). ______
Daily throughput ($v_d$) .................... ______
Transinoculation out of activated:
  Sludge vessels .................... ______
  Settling vessel .................... ______

REMARKS

| A. Controls | mg/l | |
|---|---|---|
| | TOC [1] | DOC [1] |
| Stock solution of the test material (10,000 mg active substance/litre, dilution * * * / 1,000 ml of distilled water) .................... | | |
| | | |

[1] Disagreements between TOC and DOC values point towards insufficient solubility of the test material.

All DOC values determined after membrane filtration (1.2 μm).

(c) *Data and reporting*—(1) *Treatment of results.* (i) The daily degradation rates DR are calculated according to

*Equation 1*

$$DR=\frac{T-(E-E_o)}{T}\times 100$$

(ii) For explanation of symbols, see Definitions and units, above.

(iii) These daily degradation rates are corrected for the material transfer due to the transinoculation procedure with equation 2 for a 3 hr, or equation 3 for a 6 hr, mean retention time.

*Equation 2*

$$DR_c=\frac{8}{7}DR-\frac{100}{7}$$

*Equation 3*

$$DR_c=\frac{4}{3}DR-\frac{100}{3}$$

(iv) The mean of the series of $DR_c$ values is calculated and in addition the standard deviation according to equation 4

*Equation 4*

$$^{s}DR_c=\sqrt{\frac{\sum_{i=1}^{n}(\overline{DR}_c-DR_{ci})^2}{n-1}}$$

Where:

$^{s}DR_c$=Standard deviation of the series of $DR_c$ values

$DR_c$=mean of $DR_c$ value

n=number of determinations

(v) Outliers of the $DR_c$ series are eliminated according to Nalimov under paragraph (d)(2) of this section at the 95 per cent probability level and the mean and the standard deviation of the outlier-free $DR_c$ data set recalculated. The final result is then calculated with equation 5 as

*Equation 5*

$$DR_c=DR_c\pm\frac{t_{n-1};\alpha}{\sqrt{n}}\,{}^{s}DR_c$$

Where:

$t_{n-1}$; $\alpha$=table[3] value of t for n value pairs of E and $E_o$ and statistical confidence P ($P=1-\alpha$) whereby P is set at 95 per cent.

(A) *Calculation of the results by computer.* A computer program BILAH available from Henkel A.G. (Düsseldorf, FRG) performs the above calculations automatically and is recommended for use. The program is written in Fortran IV and has been tested on a Honeywell-Bull series 6000 computer (Mark III Service). This program is designed for any continuous flow biodegradability test run with a transinoculation procedure.

(B) *Calculation of the results by pocket calculator.*The calculation described above can also be performed automatically by the programme BILAW for a pocket calculator (Texas Instruments 59 with printer). The programme instructions, the programme itself, and an example for a complete input and output are available from Henkle A.G. (Düsseldorf, FRG)

(2) *Test report.* (i) The report must contain a statement referring to the analytical accuracy with respect to the particular test material.

(ii) The result is stated as the mean with tolerance limits at the 95 percent probability level, the respective standard deviation and the number of data of the outlier-free $DR_c$ data set, e.g.

$DR_c$=98.6± 2.3) % DOC removal.

s=4.65 % DOC removal

n=18

(iii) The running-in time also constitutes pertinent information.

(iv) Some indication of the general purification such as DOC or COD removal with respect to the total organic feed, especially of the blank unit, and the effluent concentrations of the latter must be provided.

(v) All this information is summarized in the following Figure 4 report sheet:

FIGURE 4—SAMPLE REPORTING SHEET

Date of start of test:
Test material:
Test concentration:
  mg AS/l (or mg/l product)
  mg DOC/l
  mg COD/l
*Result with respect to test material*
Mean and tolerance limits at the 95 per cent probability level; standard deviation and number of determinations (outlier-free):
$\mathrm{DR}_t$= (          ) % DOC removal
s= (          ) % DOC removal
n=
$\mathrm{DR}_t$= (          ) % COD removal
s= (          ) % COD removal
n=
Working-in time: d
Evaluation period: d
*Analysis:*
Analysis of instrument response:
Analytical correctness with respect to test material:
  Percentage recovery with respect to theory at conc . . . mg DOC (or COD)/l
*General parameters:*
Mean retention time: hrs
Volume being interchanged ($v_i$): l/d
Daily throughput ($v_d$): l/d
Transinoculation out of:
  activated sludge vessel:
  settling vessel:
Origin and preparation of inoculum:
Mean organic dry matter:
  Test unit: g/l
  Blank unit: g/l
Mean DOC (COD) removal with respect to total organic load:
  Test unit: % DOC (or COD) removal
  Blank unit: % DOC (or COD) removal
Effluent of blank unit
  Concentration: mg DOC/l
  Standard deviation: mg DOC/l n:
  Concentration: mg COD/l
  Standard deviation: mg COD/l n:

*Remarks:*

(vi) Special observations, e.g. "excessive formation of bulking sludge," are to be reported under "remarks".

(vii) Finally, a degradation diagram must be provided whereby the outliers in the evaluation period are not excluded in the graph.

(d) *Literature references.* For additional background information on this test guideline the following references should be consulted:

(1) "Oxygen Demand (chemical)," *Standard Methods for the Examination of Water and Waste Water,* 14 Ed. American Public Health Association, American Water Works Association Water Pollution Control Federation, (1975) p. 550

(2) Kaiser, R., and Gottschalk G., *Elementare Tests zur Beurteilung von Messdaten,* (Bibliographisches Institut: Mannheim, Wien, Zürich, 1971) p. 18.

(3) Sachs, L., *Statistische Auswertungsmethoden,* Springer-Verlag (1971) p. 111.

(4) Fischer, W.K., Gerike, P., Holtmann, W., "Biodegradability Determinations Via Unspecific Analyses (chemical oxgen demand, dissolved organic carbon) in coupled units of the OECD Confirmatory Test. 1. The Test". *Water Research,* 9:1131-1135 (1975).

(5) Gerike, P., Fischer, W.K., and Holtmann, W. "Biodegradability Determination in Trickling Filter Units Compared with the OECD Confirmatory Test," *Water Research,* 14:753 (1980).

[50 FR 39502, Sept. 27, 1985]

## § 796.3340 Inherent biodegradability: Modified SCAS test.

(a) *Introductory information*—(1) *Prerequisites.* (i) Water solubility.

(ii) The organic carbon content of the test material must be established.

(2) *Guidance information.* (i) Information on the relative proportions of the major components of the test material will be useful in interpreting the results obtained, particularly in those cases where the result lies close to the "pass level."

(ii) Information on the toxicity of the chemical may be useful to the interpretation of low results and in the selection of appropriate test concentrations.

(3) *Qualifying statements.* (i) The method is only applicable to those organic test materials which, at the concentration used in the test,

(A) Are soluble in water (at least 20 mg dissolved organic carbon/l).

(B) Have negligible vapour pressure.

(C) Are not inhibitory to bacteria.

(D) Do not significantly adsorb on glass surfaces.

(E) Are not lost by foaming from the test solution.

(ii) This test has been found suitable by the OECD Expert Group Degradation/Accumulation for determining the inherent biodegradability of organic chemicals under aerobic conditions.

(4) *Recommendations.* Test chemicals giving a result of greater than 20 per cent loss of DOC in this test may be regarded as inherently biodegradable, whereas a result of greater than 70 per cent loss of DOC is evidence of ultimate biodegradability. The use of a compound specific analytical technique on $^{14}$C-labeled test substance may allow greater sensitivity. In these last cases a lower level may be regarded as evidence of inherent biodegradability.

(5) *Standard documents.* This Test Guideline has been based on the paper cited under paragraph (d) (1) of this section.

(b) *Method*—(1) *Introduction, purpose, scope relevance, application and limits of test.* (i)(A) The method is an adaptation of the Soap and Detergent Association semi-continuous activated sludge (SCAS) procedure for assessing the primary biodegradation of alkyl benzene sulfonate. The method involves exposure of the chemical to relatively high concentrations of microorganisms over a long time period (possibly several months). The viability of the micro-organisms is maintained over this period by daily addition of a settled sewage feed.

(B) Because of the long detention period (36 hours) and the intermittent addition of nutrients the test does not simulate those conditions experienced in a sewage treatment plant. The results obtained with the test substance indicate that it has a high biodegradation potential, and for this reason it is most useful as a test of inherent biodegradability.

(C) Since the conditions provided by the test are highly favorable to the selection and/or adaptation of mircroorganisms capable of degrading the test compound, the procedure may also be used to produce acclimatized inocula for use in other tests. The test is applicable to water soluble, nonvolatile, organic chemicals that are not inhibitory to bacteria at the test concentration.

(ii) *Reference substances.* In some cases when investigating a new substance reference substances may be useful; however, specific reference substances cannot yet be recommended. Data on several compounds used in ring tests are provided (see Annex) primarily so that calibration of the method may be performed from time to time and to permit comparison of results when another method is employed.

(iii) *Principle of the test method.* (A) Activated sludge from a sewage treatment plant is placed in an aeration (SCAS) unit. The test compound and settled domestic sewage are added, and the mixture is aerated for 23 hours. The aeration is then stopped, the sludge allowed to settle and the supernatant liquor is removed. The sludge remaining in the aeration chamber is then mixed with a further aliquot of test compound and sewage and the cycle is repeated.

(B) Biodegradation is established by determination of the dissolved organic carbon content of the supernatant liquor. This value is compared with that found for the liquor obtained from a control tube dosed with settled sewage only.

(iv) *Quality criteria*—(A) *Reproducibility.* The reproducibility of this modification of the method based on removal of dissolved organic carbon has not yet been established. When primary biodegradation is considered, very precise data is obtained for materials that are extensively degraded. The results reported in reference (1) suggest 95 per cent confidence limits of less than ±3 per cent, and this includes interlaboratory tests. As would be expected, wider confidence limits are obtained for less biodegradable materials.

(B) *Sensitivity.* The sensitivity of the method largely depends on the precision of the determination of dissolved organic carbon and the level of test compound in the liquor at the start of each cycle. At the end of the aeration period about 10 mg/litre of dissolved organic carbon remain in the supernatant liquor of the control experiment. Assuming that the dissolved organic carbon determination is within ±5 per cent and a level of 20 mg/litre of carbon as test material is added at the start of the aearation period, then the assessment of the extent of biodegradation should be within ±6 per cent for the range 80-100 per cent biodegradation.

(C) *Specificity.* The method is applicable to any non-volatile, water soluble, organic compound.

(D) *Possibility of standardization.* Since the method uses a feed of real settled sewage, absolute standardization is not possible unless this feed were replaced by an artificial one. However, since the method is designed to give an indication of the biodegradability potential of a chemical and is not a simulation test, such standardization is unnecessary.

(E) *Possibility of automation.* Automation of this method would be possible but would be expensive. As the method is not labor intensive, the exercise would offer few advantages.

(2) *Description of the test procedure*—(i) *Preparations.* (A) The aeration units are cleaned and fixed in a suitable support. The air inlet tubes are connected to the supply manifold. A small laboratory scale air compressor is used to aerate the units, and the air is presaturated with water to reduce evaporation losses from the units.

(B) A sample of mixed liquor from an activated sludge plant treating predominantly domestic sewage is obtained. Approximately 150 ml of the mixed liquor are required for each aeration unit.

(C) The organic carbon analyzer is calibrated using potassium hydrogen phthalate.

(D) Stock solutions of the test compounds are prepared: the concentration normally required is 400 mg/litre as organic carbon which gives a test compound concentration of 20 mg/litre carbon at the start of each aeration cycle if no biogradegradation is occurring.

(E) The organic carbon content of the stock solutions is measured.

(ii) *Test conditions.* A high concentration of aerobic micro-organisms is used, and the effective detention period is 36 hours. The carbonaceous material in the sewage feed is oxidized extensively within 8 hours of the start of each aeration cycle. Thereafter, the sludge respires endogeneously for the remainder of the aeration period, during which time the only available substrate is the test compound unless this is also readily metabolized. These features, combined with daily reinoculation of the test when domestic sewage is used as the medium, provide highly favorable conditions for both acclimatization and biodegradation.

(iii) *Performance of the test.* (A) A sample of mixed liquor from a suitable activated sludge plant is obtained and aerated during transportation to the laboratory. Each aeration unit is filled with 150 ml of mixed liquor and the aeration is started. After 23 hours, aeration is stopped, and the sludge is allowed to settle for 45 minutes. The tap is opened and 100 ml of the supernatant liquor withdrawn. A sample of settled domestic sewage is obtained immediately before use, and 100 ml are added to the sludge remaining in each aeration unit. Aeration is started anew. At this stage no test materials are added, and the units are fed daily with domestic sewage only until a clear supernatant liquor is obtained on settling. This usually takes up to two weeks, by which time the dissolved organic carbon in the supernatant liquor at the end of each aeration cycle should be less than 12 mg/litre.

(B) At the end of this period the individual settled sludges are mixed, and 50 ml of the resulting composite sludge are added to each unit.

(C) 100 ml of settled sewage are added to the control units and 95 ml plus 5 ml of the appropriate test compound stock solution (400 mg/l) to the test units. Aeration is started again and continued for 23 hours. The sludge is then allowed to settle for 45 minutes and the supernatant drawn off and analzed for dissolved organic carbon content.

(D) The fill and draw procedure under paragraph (b)(2)(iii)(A) of this section is repeated daily throughout the test.

(E) Before settling it may be necessary to clean the walls of the units to prevent the accumulation of solids above the level of the liquid. A separate scraper or brush is used for each [illegible] to prevent cross contamination.

(F) Ideally, the dissolved organic carbon in the supernatant liquors is determined daily, although less frequent analysis is permissible. Before analysis the liquors are filtered through washed 0.45 μm membrane filters and certifuged. Temperature of the sample must not exceed 40° C while it is in the centrifuge.

(G) The length of the test for compounds, showing little or no biodegradation is indeterminate, but experience suggests that this should be at least 12 weeks.

(c) *Data and reporting*—(1) *Treatment of the results.* (i) The dissolved organic carbon results in the supernatant liquors of the test units and the control units are plotted against time. As biodegradation is achieved the level found in the test will approach that found in the control. Once the difference between the two levels is found to be constant over three consecutive measurements, 3 further measurements are made and the percentage biodegradation of the test compound is calculated by the following equation:

$$\% \text{ biodegradation} = \frac{100\,[O_T-(O_t-O_c)]}{O_T}$$

Where:

$O_T$=concentration of test compound as organic carbon added to the settled sewage at the start of the aeration period.

$O_t$=concentration of dissolved organic carbon found in the supernatant liquor of the test at the end of the aeration period.

$O_c$=concentration of dissolved organic carbon found in the supernatant liquor of the control

(ii) The level of biodegradation is therefore the percentage elimination of organic carbon, under the following Figure 1:

FIGURE 1—EXAMPLES OF RESULTS OF SCAS TEST ON VARIOUS COMPOUNDS USED IN THE OECD/EEC RING TEST

| Test compound | $O_T$ (mg/l) | $O_t-O_c$(mg/l) | Percentage biodegradation/bioelimination |
|---|---|---|---|
| 4-acetyl aminobenzene sulphonate | 17.2 | 2.0 | 85 |
| Tetra propylene benzene sulphonate | 17.3 | 8.4 | 51.4 |
| 4-nitrophenol | 16.9 | 0.8 | 95.3 |
| Diethylene glycol | 16.5 | 0.2 | 98.8 |
| Aniline | 16.9 | 1.7 | 95.9 |

Duration of test 40 days.

RESULTS FOUND FOR CYCLOPENTANE TETRA CARBOXYLATE

| $O_T$ (mg/l) | $(O_t-O_c)$ (mg/l) | Percentage biodegradation/bioelimination |
|---|---|---|
| 17.9 | 3.2 | 81.1 |

Duration of test 120 days.

(iii) If from the outset there is no difference between the control and the test, or the difference between the two remains constant at a level less than would be expected if no degradation had taken place, further tests are necessary to distinguish between biodegradation and adsorption. This may be done by using the supernatant liquors as a source of inoculum for tests such as the Sturm or the Closed Bottle Tests under §§ 796.3200 and 796.3260.

(d) *Literature references.* For additional background information on this test guideline the following references should be consulted:

(1) "A Procedure and Standards for the Determination of the Biodegradability of Alkyl Benzene Sulphonate and Linear Alkylate Sulphonate", *Journal of the American Chemical Society*, Vol. 42 (1965), p. 986.

(2) [Reserved]

[50 FR 39506, Sept. 27, 1985]

**§ 796.3360 Inherent biodegradability: Modified Zahn-Wellens test.**

(a) *Introductory information*—(1) *Prerequisite.* Water solubility.

(2) *Guidance information.* (i) Information on the relative proportions of the major components of the test material will be useful in interpreting the results obtained, particularly in those cases where the result lies close to the "pass level".

(ii) Information on the toxicity of the chemical may be useful to the interpretation of low results and in the selection of appropriate test concentrations.

(3) *Qualifying statements.* (i) The method is only applicable to those organic test materials which, at the concentration used in the test:

(A) Are soluble in water to the extent necessary for the preparation of the test solutions.

(B) Have negligible vapour pressure.

(C) Are not inhibitory to bacteria.

(D) Do not significantly adsorb on glass surfaces.

(E) Are not lost by foaming from the test solution.

(ii) This test has been found suitable by the OECD Expert Group "Degradation/Accumulation" for determining the inherent biodegradability of organic chemicals under aerobic conditions. It has been tested in the OECD Laboratory Intercomparison Test Program (1978–1980).

(4) *Recommendations.* Test chemicals giving a result of greater than 20 percent loss of DOC (dissolved organic carbon) in this test may be regarded as inherently biodegradable, whereas a result of greater than 70 per cent loss of DOC is evidence of ultimate biodegradability. The use of a compound specific analytical technique on $^{14}$C-labeled test substances may allow greater sensitivity. In these last cases a lower level may be regarded as evidence of inherent biodegradability.

(5) *Standard documents.* This Test Guideline is based on a modification of the method cited under paragraph (d) (1) and (2) of this section.

(b) *Method*—(1) *Introduction, purpose, scope, relevance, application and limits of test.* (i)(A) The static test is a simple, reproducible method for evaluating the ultimate biodegradability of organic substances in water by microorganisms in an aerobic milieu.

(B) The static method is limited t the examination of the biodegradability of water soluble, non-volatile organic compounds. The compounds t be studied are used in concentration corresponding to DOC-values in the range of 50–400 mg/litre or COD-values in the range of 100–1000 mg/litre (DOC=dissolved organic carbon; COD=chemical oxygen demand).

(C) These relatively high concentrations have the advantage of analytical reliability. Compounds with toxic properties may delay or inhibit the degradation process.

(ii) *Definition and units.* (A) The amount of degradation attained at the end of the test is reported as the "Biodegradability in the Static Test":

$$D_T(\%) = 1 - \frac{(C - C_B)}{C_A} \cdot 100$$

$D_T$=biodegradation (%) at time T

$C_A$=initial value (DOC or COD values in the test mixture calculated from the DOC or COD values in the stock solution, [mg/l]

$C_T$=DOC or COD values at time of sampling, [mg/l]

$C_B$=DOC or COD value of the blank, [mg/l]

(B) The degradation rates are rounded to the nearest full percent.

(C) Percentage degradation is stated as the percentage DOC (or COD) removal of the tested substance.

(iii) *Reference substances.* In some cases when investigating a new substance reference substances may be useful, however specific reference substances cannot yet be recommended.

(iv) *Principle of the test method.* Activated sludge, mineral nutrients and the test material as the sole carbon source in an aqueous solution are placed together in a 1–4 litre glass vessel equipped with an agitator and an aerator. The mixture is agitated and aerated at 22 °C (±3°) under diffuse illumination or in a dark room for up to 28 days. The degradation process is monitored by determination of the DOC (or COD) values in the filtered solution at daily or other appropriate regular time intervals. The ratio of eliminated DOC (or COD) after each interval to the value at the start is expressed as percentage biodegradation and serves as the measure for the rate of degradation at this time. The result is plotted versus time to give the biodegradation curve.

(v) *Quality criteria*—(A) *Reproducibility.* Reproducibility has been proven in ring tests. Detection of <20 per cent; >20 to <70 per cent; >70 per cent DOC-removal as required for testing inherent biodegradability is possible.

(B) *Sensitivity.* The limits for sensitivity are given by the sensitivity of the carbon analysis (0.5–1 mg C/l) and the COD-analysis (5–10 mg $O_2$/l).

(C) *Specificity.* Applicable for tests with water soluble (>100 mg/l), nonvolatile organic substances.

(D) *Possibility of standardization.* Standardization is possible.

(E) *Possibility of automation.* Automation of analysis is possible.

(2) *Description of the test procedure*—(i) *Preparations*—(A) *Reagents.* (*1*) Test water: drinking water with an organic-carbon content <5 mg/l. The concentration of calcium and magnesium ions together must not exceed 2.7 mole/l; otherwise adequate dilution with deionised or distilled water is required.

(*2*) Sulfuric acid, analytic reagent (A.R.) 50 g/l.

(*3*) Sodium hydroxide solution, A.R., 40 g/l.

(*4*) Mineral nutrient solution: dissolve in one litre deionized water:

38.5 g ammonium chloride, $NH_4Cl$, A.R.

33.4 g sodium dihydrogenphosphate, $NaH_2PO_4 \cdot 2H_2O$, A.R.

8.5 g potassium dihydrogenphosphate, $KH_2PO_4$, A.R.

21.75 g di-potassium mono-hydrogenphosphate $K_2HPO_4$, A.R.

The mixture serves both as a nutrient compound and as a buffering system.

(B) *Apparatus.* (*1*) Glass vessels with a volume of 1–4 litre (e.g., cylindrical vessels).

(*2*) Agitator with a glass or metal stirrer on a suitable shaft. (The stirrer should rotate about 5 to 10 cm above the bottom of the vessel.) A magnetic stirrer with a 7–10 cm long rod can be used instead.

(*3*) Glass tube of 2–4 mm inner diameter to introduce air. The opening of the tube should be about 1 cm above the bottom of the vessel.

(*4*) Centrifuge (at least 2000 rpm).

(*5*) pH-meter.

(*6*) $O_2$-measuring instrument.

(*7*) Paper filters.

(*8*) Membrane filtration apparatus.

(*9*) Membrane filters, pore size 0.2 μm.

(*10*) Analytical equipment for determining organic carbon content and chemical oxygen demand.

(C) *Preparation of the inoculum.* (*1*) Activated sludge from a biological treatment plant is washed by (repeatedly) centrifuging or settling with testwater under paragraph (b)(2)(i)(B)(*4*) of this section. The activated sludge must be in an appropriate condition. Such sludge is available from a properly working sewage treatment plant. To get as many possible different species or strains of bacteria in special cases it may be preferred to make a mixture from different sources (e.g. different treatment plants, soil extracts, river waters, etc.). The mixture is to be treated as described under paragraph (b)(2)(i)(A) of this section.

(*2*) For checking the activity of the activated sludge see Functional control, under paragraph (b)(2)(ii)(E)(*1*) of this section.

(D) *Preparation of the test solutions.* (*1*) To the test vessel add 500 ml of test water 2.5 ml/litre mineral nutrient solution and activated sludge in an amount corresponding to 0.2–1.0 g/l (normally 0.2 or 1.0 g/l (The lower concentrations: 0.2 g dry matter of activated sludge/litre and a DOC-concentration of 50 mg/l are introduced to make the test instructions compatible with the instructions of the EMPA-Test (Switzerland) under paragraph (d)(4) of this section.)) dry matter in the final mixture. Add sufficient stock solution of the test material or of the waste water to be tested that a DOC concentration of 50–400 mg/l (normally 50 or 400 mg/l (The lower concentrations: 0.2 g dry matter of activated sludge/litre and a DOC-concentration of 50 mg/l are introduced to make the test instructions compatible with the instructions of the EMPA-Test (Switzerland) under paragraph(d)(4) of this section.)) results in the final mixture. The corresponding COD-values are 100-1000 mg/l. Make up with test water to a total volume of 1-4 litres. The total volume to be chosen is dependent on the number of samples to be taken for DOC or COD determination and the volumes necessary for the analytical procedure.

(*2*) Normally a volume of 2 litres can be regarded as satisfactory.

(*3*) At least one control vessel (blank) is set up to run in parallel with each test series; it contains only activated sludge and mineral nutrient solution made up with test water to the same total volume as in the test vessels.

(*4*) Note: Before starting the test it is advisable to make certain with appropriate methods that no inhibition occurs at the chosen concentration of test material. Run the test with a smaller concentration if an inhibitory effect is found.

(ii) *Performance of the test.* (A) The test vessels are agitated with magnetic stirrers or screw propellers under diffuse illumination or in a dark room at 22 °C (±3°). Aeration is accomplished by compressed air cleaned by a cotton wool strainer and a wash bottle if necessary. It must be ensured that the sludge does not settle and the oxygen concentration does not fall below 2 mg/l.

(B) The pH-value must be checked at regular intervals (for example daily) and adjusted to pH 7-8 with NaOH or $H_2SO_4$, if necessary.

(C) Losses from evaporation are made up just before each sampling with deionized or distilled water in the required amounts. The best procedure is to mark the liquid level on the vessel before starting the test and after each sampling (without aeration and stirring). The first samples are always taken 3 hours after the start of the test in order to detect adsorption of test material by the activated sludge.

(D) The elimination of the test material is followed by DOC- or COD-determinations made daily or at some other regular interval. The samples from the test vessel and the blank are filtered through a carefully wa paper filter. The first 5 ml of test so tion-filtrate are returned to the t vessel. Sludges difficult to filter m be removed previously by centrifu tion. DOC and COD determinatio are made at least in duplicate. T test is run for up to 28 days.

(E) Note: Turbid remaining sampl are filtered through membrane filte The membrane filters must not relea or adsorb any organic material. Othe wise they are to be purified by boilin in deionised water previously. For pr cedure in connection with adaptatio processes, see paragrap (b)(2)(ii)(E)(*2*) of this section.

(*1*) *Functional control.* A vessel wit a known substance should be run par allel with each test series in order t check the functional capacity of th activated sludge. For this purpos compounds, such as diethyleneglycol sodium benzoate, and aniline, are rec ommended.

(*2*) *Adaptation.* (*i*) If analyses are carried out at relatively short interval (e.g. daily), adaptation can be clearly recognized from the degradation curve (see Figure 2).

(*ii*) If the adaptation occurs in the final days of the test time, the test time can be prolonged until the degradation is finished.

(*3*) Note: If a broader knowledge of the behaviour of the adapted sludge is needed, the same activated sludge is exposed once again to the same test material in accordance with the following procedure:

(*i*) Switch off the agitator and the aerator and allow the activated sludge to settle. Draw off the supernatant liquid, fill up to 2 litres with test water, stir for 15 minutes and allow to settle again. After the supernatant liquid is drawn off again use the remaining sludge to repeat the test with the same test material in accordance with Preparation of test solutions and Performance of the test, above. The activated sludge can also be isolated by centrifuging instead of settling.

(*ii*) The adapted sludge may be mixed with fresh sludge to a total amount of 0.2-1 g dry weight/litre.

(iii) *Analytical means.* (A) Normally samples are filtered through a carefully washed paper filter (for washing use deionised water).

(B) Samples which remain are filtered through membrane filters (0.2 μm, diameter 25 mm). Membrane filters are suitable if it is assured that they neither release nor adsorb organic compounds. Otherwise the membrane filters must be purified from soluble organic material by boiling them 3 times in deionized water. The purified filters may be stored in water.

(C) The DOC concentration is determined twice in the sample filtrates (the first 5 ml are discarded) by means of the TOC instrument. If the filtrate cannot be analyzed on the same day, it must be stored in the refrigerator until the next day. Longer storage cannot be recommended.

(D) The COD concentration is determined in the sample filtrates with a COD analytical set up by the procedure described under paragraph (d)(3) of this section.

(c) *Data and reporting*—(1) *Treatment of results*. (i) DOC and COD concentrations are determined at least in duplicate in the samples according to Performance of the Test and Analytical means, above. The degradation at the time T is calculated according to the formula (with definitions) given under Definitions and units, under paragraph (b)(1)(ii) of this section.

(ii) The degradation rates are rounded to the nearest full percent. The amount of degradation attained at the end of the test is reported as the "Biodegradability in the Static Test".

(iii) Note: If complete degradation is attained before the test time is over and this result is confirmed by a second analysis on the next day, the test can be concluded.

(2) *Test report*. (i) The test report comprises information about:

(A) The test substance (name, structure, impurities, solubility, concentration, etc.).

(B) The inoculum (sampling of the inoculum, concentration, status of adaptation).

(C) The kind of analysis.

(D) The toxicity evaluations.

(E) The functional control (calibration compound).

(ii) The test results at different sampling times, are seen in the following example:

EVALUATION EXAMPLE

Organic compound: 4-Ethoxybenzoic acid
Theoretical test concentration: 600 mg/l
Theoretical DOC: 390 mg/l
Inoculum: Treatment plant of HOECHST AG in Frankfurt/M.-Höchst
Concentration: 1 g dry material/litre
Adaptation status: not adapted
Analysis: DOC-determination
Amount of sample: 3ml
Functional control: Diethyleneglycol
Toxicity of compound: No toxic effects below 1000 ppm (Gärröhrchentest)

| | *Functional control* | | | | *Test compound* | | |
|---|---|---|---|---|---|---|---|
| Sampling blank time | DOC [1] mg/l | DOC [1] mg/l | DOC net mg/l | Degrad. % | DOC [1] mg/l | DOC [1] net mg/l | Degrad. % |
| 0 | | | 300 | | | 390 | |
| 3 h | 4.0 | 298 | 294 | 2 | 371.6 | 367.6 | 6 |
| 1 d | 6.1 | 288.3 | 282.2 | 6 | 373.3 | 367.2 | 6 |
| 2 d | 5.0 | 281.2 | 276.2 | 8 | 360.0 | 355.0 | 9 |
| 5 d | 6.3 | 270.5 | 264.2 | 12 | 193.8 | 187.5 | 52 |
| 6 d | 7.4 | 253.3 | 245.9 | 18 | 143.9 | 136.5 | 65 |
| 7 d | 11.3 | 212.5 | 201.2 | 33 | 104.5 | 93.2 | 76 |
| 8 d | 7.8 | 142.5 | 134.7 | 55 | 58.9 | 51.1 | 87 |
| 9 d | 7.0 | 35.0 | 28.0 | 91 | 18.1 | 11.1 | 97 |
| 10 d | 18.0 | 37.0 | 19.0 | 94 | 20.0 | 2.0 | 99 |

[1] Mean values of triplicate determinations.

(iii) A second part of the test report is made up by the biodegradation curve in the following Figures 1 and 2:

FIGURE 1—STATIC TEST—EXAMPLES OF BIODEGRADATION CURVES

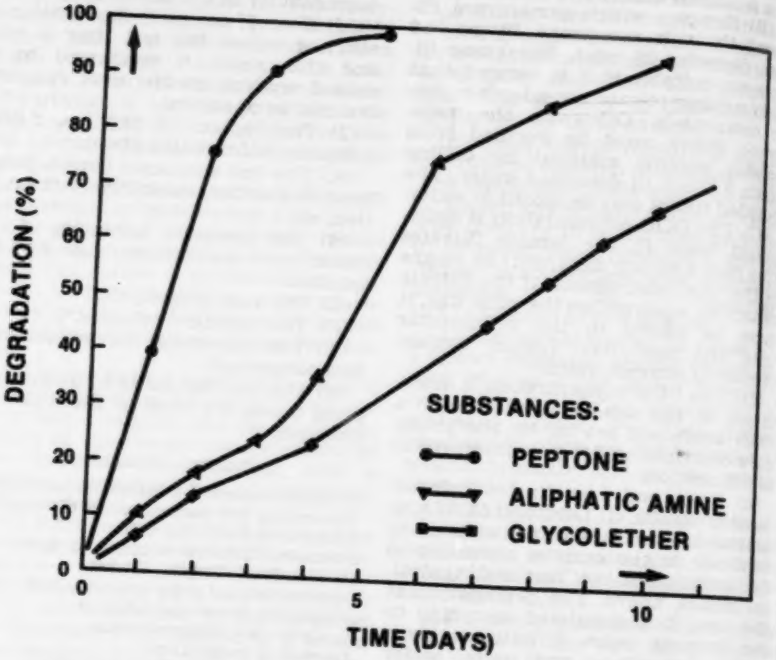
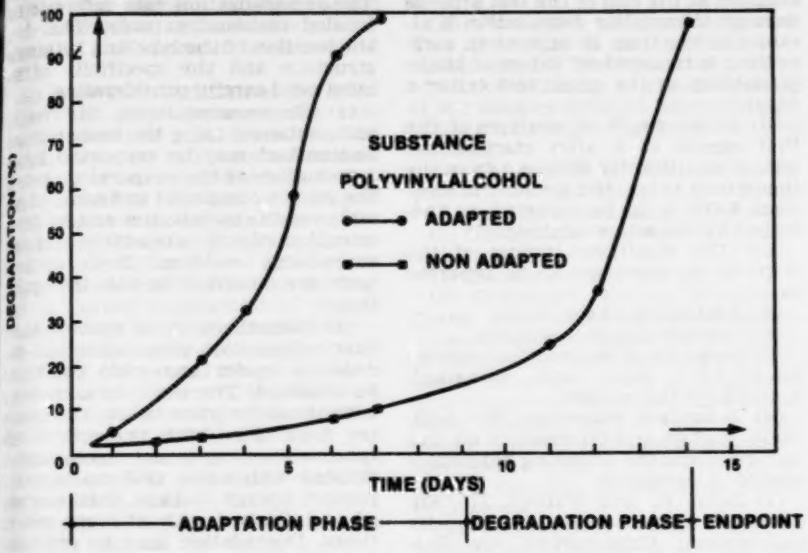


FIGURE 2—STATIC TEST—EXAMPLES OF SLUDGE ADAPTATION



(3) *Interpretation/evaluation of results.* (i) The degree of biodegradation attained at the end of the test after 28 days or, if complete degradation is attained in less than 28 days, at an earlier time, is reported as "inherent biodegradability in the static test (after x days)."

(ii) If the result of analysis of the first sample (3 h after starting the test) is significantly different from the theoretical value, the amount of deficient DOC is to be reported as "adsorbed by the activated sludge."

(iii) The significant points of the degradation curve are to be reported as

(A) Adaptation-phase (days).

(B) Degradation-phase (days).

(C) Endpoint of degradation reached after * * * days (see paragraph (c)(2)(iii) of this section.

(d) *Literature references.* For additional background information on this test guideline the following references should be consulted:

(1) Zahn, R. und Wellens, H. "Ein einfaches Verfahren zur Prüfung der biologischen Abbaubarkeit von Produkten und Abwasserinhaltsstoffen," *Chemiker-Zeitung* 98 (1974) p. 228–232

(2) Umweltbundesamt: OECD-Ring-Test Programme on Detecting Biodegradability of Chemicals in Water, Berlin July 31, Test prescription No. 4, 1978.

(3) *Standard Methods for the Examination of Water and Waste Water,* 13th ed., (No. 220), (American Public Health Association, 1971) pp. 495–499.

(4) Schefer, W., "Bestimmung den biologischen Eliminierbackeit von Abwasser-Inhaltsstoffen," *Forum-Stadte-Hygiene* 110 (1978).

[50 FR 39508, Sept. 27, 1985]

## § 796.3400 Inherent biodegradability in soil.

(a) *Introductory information*—(1) *Prerequisites.* $^{14}$C-labeled material is required.

(2) *Guidance information.* Information on the toxicity of the test compound is useful for the interpretation of the data obtained. The concentration of the test compound can then be adapted to this information.

(3) *Qualifying statements.* The test is applicable to volatile or non-volatile, soluble or insoluble compounds whic are not inhibitory to micro-organism The mineralization rate refers to th labeled carbonation only. Therefore the location of the labelling within th structure and the specificity of th label need careful consideration.

(4) *Recommendations.* (i) The results obtained using the basic mineralization test may be supported by determination of the evaporation rate of the parent compound and some of possible volatile metabolites and by determination of soil extractable and non-extractable residues. Both optional tests are described in this test guideline.

(ii) Sometimes it is recommended that information about chemical degradation under anaerobic conditions be obtained. Therefore, in accordance with the description below, the biometer flask filled with the soil sample (preconditioning is not necessary), is flooded with water (2–3 cm layer) to protect against leakage, then evacuated and flushed with nitrogen several times. Degradation may be evaluated by means of measurements of methane gas and analysis of both water and soil for $^{14}$C-content.

(5) *Standard documents.* This test guideline is based on the method cited in paragraph (d)(1) of this section.

(b) *Method*—(1) *Introduction, purpose, scope, relevance, application and limits of test.* (i) The method described in this test guideline is designed for the evaluation of the mineralization rate of a $^{14}$C-labeled compound in soil. The method is applicable to volatile or non-volatile, soluble or insoluble compounds which are not inhibitory to micro-organisms.

(ii) *Definitions and units.* (A) Soil is a mixture of mineral and organic chemical constituents, the latter containing compounds of high carbon and nitrogen content and of high molecular weights, animated by small (mostly micro-) organisms. Soil may be handled in two states:

(*1*) Undisturbed, as it has grown with time, in characteristic layers of a variety of soil types,

(*2*) Disturbed, as it is usually sampled by digging and used in the test described here.

(B) Mineralisation (in this context) means extensive degradation of a molecule during which a labelled carbon atom is oxidised quantitatively with release of the appropriate amount of $^{14}CO_2$.

(iii) *Reference substances.* In some cases when investigating a new substance reference substances may be useful; however, reference substances cannot yet be recommended. Reference substances need not be employed in all cases when investigating a new substance. They may primarily be used so that calibration of the method may be performed from time to time and to permit comparison of results when another method is employed.

(iv) *Principle of the test method*—(A) *Basic test.* (*1*) A small sample of soil is treated with the $^{14}$C-labeled test chemical in a biometer flask apparatus. Release of $^{14}CO_2$ from the test chemical is measured by means of alkali absorption and liquid scintillation counting.

(*2*) Optional experiments include the following tests.

(B) *Evaporation test.* When testing chemicals of a vapour pressure higher than 0.0133 Pa, a polyurethane foam plug is placed into the biometer flask apparatus to absorb the labelled volatile part of the parent compound and volatile metabolites for liquid scintillation counting.

(C) *Residue test.* At the point of 50 per cent mineralization the test soil may be extracted. The extractable portion of the compound, and its metabolites remaining in the soil, may be determined by liquid scintillation counting. Furthermore, data on the bound residue part may be obtained by measuring the $^{14}CO_2$ released after combustion of the soil.

(v) *Quality criteria*—(A) *Reproducibility.* Reproducibility is good if standard conditions, especially preconditioning of the soil, are strictly observed.

(B) *Sensitivity.* The evaluation of sensitivity is not relevant because a moderate amount as 37-185 kBq (1-5 μCi) of $^{14}$C-labelled test chemicals is used for each experiment.

(C) *Specificity.* The method is only applicable if $^{14}$C-labeled test chemicals are available. The specificity is very good.

(D) *Possibility of standardization.* This procedure is standardized to a limited extent. The limitation is related to the difficulty of standardization of soil samples between laboratories.

(E) *Possibility of automation.* Not foreseen.

(2) *Description of the test procedure*—(i) *Preparations*—(A) *Equipment.* (*1*) Liquid scintillation counter.

(*2*) Oxidizer for combustion of radioactive material.

(*3*) Ultrasonic bath, 500 ml.

(*4*) Glassware: 250 ml Erlenmeyer flasks fused to 50 ml round bottom tubes (biometer flasks, see Figure 1); 25 ml syringes (e.g. Luer-lock); syringe needle 15 gauge, 15 cm in length; 100 μl syringes (e.g. Hamilton); 25 ml graduated cylinders with stopper; 1 ml pipettes; soxhlet extraction apparatus; scintillation vials; polyurethane plugs, 30 mm diameter, 30 mm length, density 16 kg/m$^3$.

(B) *Reagents*—(*1*) *Test substance:* $^{14}$C-labelled compounds are dissolved in water or acetone to give radioactivity of 37-185 KBq (≈1−5 μCi)/100 μl. Using unlabelled material this solution is made up to the required concentration (e.g. 0.5 mg/100 μl≈10 mg/kg soil, or depending on the toxicity of the substance).

(*2*) *Chemicals.* (*i*) KOH, analytical grade, 0.1 N solution.

(*ii*) Acetone, analytical grade.

(*iii*) Methanol, analytical grade (for optional tests).

(*iv*) n-Hexane, analytical grade (for optional test).

(*v*) Ascarite (A.H. Thomas Co. Philadelphia or equivalent).

(*vi*) Scintillation cocktail.

(*3*) *Soil.* (*i*) Alfisol: pH between 5.5 and 6.5 organic C content between 1 and 1.5 percent clay content (i.e. particles <0.002 mm in diameter) between 10 and 20 percent cation exchange capacity between 10 and 15 mval.

(*ii*) Spodosol: pH between 4.0 and 5.0 organic C content between 1.5 and 3.5 per cent clay content <per cent cation exchange capacity <10 mval.

(*iii*) Entisol: pH between 6.6 and 8.0 organic C content between 1 and 4 per cent clay content between 11 and 25 per cent cation exchange capacity >10 mval.

In special cases it is recommended that two additional soils be used: one with high silt-fraction (Diameter between 0.002 and 0.063 mm) content, the other with a high clay content (30 per cent). Air dried test soil stored at +4 °C is remoisturized to 40 per cent maximum water capacity. After incubation for 2 weeks at 22 °C+2 °C in the dark it is ready for the experiments.

(ii) *Test conditions*—(A) *Test temperature.* During the whole test period the flasks are incubated in the dark at 22 °C±2 °C

(B) *Soil characterization data.* (*1*) for determination of the pH value of the soil for selecting the soil type, 10 g air-dried soil are suspended in 25 ml 0.01 M $CaCl_2$.

(*2*) After standing overnight the sample is disturbed once more and measured in a potentiometric apparatus with a 0.1 M KCl electrode. Immediately before the measurement the instrument must be calibrated with two standard solutions within the measuring range of the sample values expected.

(*3*) For determination of the organic carbon content of the soil for selecting the soil type, 1.0 g air-dried soil is heated with 15 ml 2M $K_2Cr_2O_7$ and 20 ml $H_2SO_4$ (analytical reagent, $\rho c=1.84$ g/cm$^3$) at 145-155 °C for 15 minutes. After cooling to room temperature sample volume is made up to 150 ml with distilled water. A 20 ml aliquot is measured spectrophotometrically, after centrifuging, in a 1 cm cuvette at 590 nm compared to distilled water. The self-destroying property of the $K_2Cr_2O_7$ reagent must be determined by two blank samples. Calculation is conducted using the following equation:

$$C= \frac{\cdot 1000 \cdot v \cdot E_2(E_x-\alpha_2 \cdot c)}{e \cdot E_1 \cdot (\alpha_1-\alpha_2 \cdot F)}$$

Where:

C=carbon content (%)
V=gross volume (ml)
$E_1$=equivalent weight of $Cr_2O_3$ (25.332)
$E_2$=equivalent weight of carbon (3.0028)
$E_x$=extinction at 590 nm and 1 cm laye thickness
F=factor calculating $K_2Cr_2O_7$ from $Cr_2O_3$
c=concentration of Cr (g) per 100 m (=1.9356)
e=sample weight (mg)
$\alpha_1$=extinction coefficient of Cr (III) $\alpha_1$ is a average value from five single determi nations for the calibration curve, eac obtained by division of $E_x$ by the amoun of $Cr_2O_3$ (in g)
$\alpha_2$=extinction coefficient of Cr (VI) $\alpha_2$ is a average value from two single estimations, each obtained by division of $E_x$ by the respective amounts of $K_2Cr_2O_7$.

(*4*) For determination of particle size of the soil for selecting the soil type, 10.0 g air-dried soil are reacted with 100 ml $H_2O_2$ (15 per cent $^v/_v$ for 15 hours, then heated until $CO_2$ evolution is complete. Afterwards the suspension is left to stand overnight with 25 ml 0.4 N $Na_4P_2O_7$, then water is added to make it up to 250 ml and the solution is sieved through a mesh of 0.2 mm width. The portion >0.2 mm is fractionated further by sieving. The smaller particles (silty fractions) are fractionated by homogenous partitioning of the particles in the aqueous medium, which is made up to 1000 ml with water in an elutriating cylinder.

(*5*) 10 ml portions are removed by pipette from various heights of the cylinder after different sedimentation times; measurement of the dry weights of the suspended material in these portions yields the particle composition according to the following scheme:

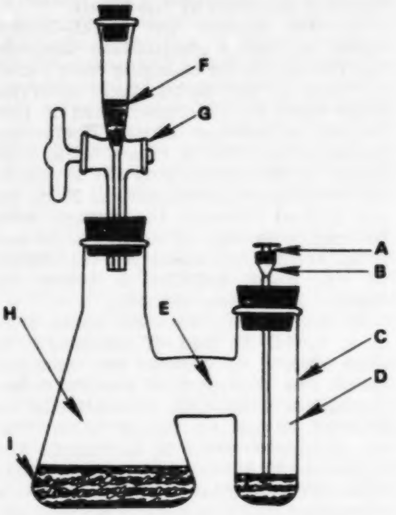
| Particle fraction diameter (mm) | Dipping depth (cm) | | | |
|---|---|---|---|---|
| | 20 | 15 | 10 | 5 |
| <0.002 | ...... | ...... | 7h 45m | 3h 52m |
| <0.0063 | 1h 33m 49s | 1h 10m 52s | 46m 55s | 23m 27s |
| <0.02 | 9m 19s | 6m 59s | 4m 39s | 2m 20s |
| <0.063 | 59s | 42s | ...... | ...... |

(*6*) For determination of the cation exchange capacity of the soil, in order to select the soil type, a glass column 15 cm in length and 30 mm inner diameter is reduced in diameter at one end like a funnel. This side is stuffed with filter wool. About 1 cm quartz sand is strewn on the wool, followed by 10.0 g air-dried test soil, which is in turn covered by about 1 cm quartz sand. Above these layers comes 40 ml of a mixed solution [consisting of 100 g triethanolamine in 2 l water (adjusted to ph 8.1 with HCl) plus 100 g $BaCl_2 \cdot 2H_2O$ in 2 litres). After 1 h the solution is collected in an Erlenmyer flask of 250 ml. The procedure is then repeated. In addition, 40 ml of a solution of 25 g $BaCl_2 \cdot 2H_2O$ in 1 l are poured into the column.

(*7*) After standing overnight, this solution is also collected and the column is washed with 100 ml water. The combined eluates are titrated against HCl (bromocresol green plus methyl red as indicators) to measure $H^+$, $Ca^{2+}$, $K^+$, $Na^+$. For the determination of $Ba^{2+}$ the column is leached in a similar mannner with 200 ml of 20 g $MgCl_2 \cdot 6H_2O$ in 1-litre water. This cation is determined by flame absorption spectrophotometry. The cation exchange capacity is expressed as the sum of all the cation equivalents sorbed by 100 g soil.

(iii) *Performance of test*—(A) *Basic test.* (*1*) Fifty grams of soil (dry weight basis) are placed into each Erlenmeyer part (H) of the biometer flask (see the following Figure 1). 100 μl of the radioactive test solution are added in 50 drops over the whole soil surface (I) of each flask. Then, the soil is carefully mixed with a Pasteur pipette (from which the lower part is cut off) and left in the flask.

FIGURE 1—TEST FLASK



(*2*) In addition, an equivalent volume of test solution is placed in a 100 ml volumetric flask for direct determination of the added radioactivity.

(*3*) The biometer flask is closed with a teflon-coated silicon rubber stopper through which an Ascarite filter (F) is inserted. The filter (F) is provided with a stopper and stopcock (G). The side tube (C) is sealed with a teflon stopper pierced by a 15-gauge needle (B), 15 cm long. The needle (B) is capped by a silicone rubber stopper (A), and its tip at (D) is covered with a short length of silicone tubing that remains in contact with the base of the side tube (C).

(*4*) The unit is charged by injecting 10 ml of alkali solution into the side tube (C) in the following manner: the small stopper (A) is replaced by a calibrated Luer lock syringe containing 0.1 N KOH; then the filter stopper on (F) is removed and the stopcock (G) is opened; the alkali solution is introduced through the needle (B) into the side tube (C); then the stopcock is closed; the syringe is removed; the small stopper (A) and filter stopper on (F) are then returned to their initial positions. The $^{14}C$-carbon dioxide produced is absorbed by the alkali.

(*5*) To recover the $^{14}CO_2$-loaded alkali for liquid scintillation analysis, the procedure for charging each parallel unit at increasing time intervals after start of the experiment is performed in reverse order. Thereafter the side tube (C) is rinsed with 5 ml alkali. Before recharging the side tube (C) fresh alkali, 3 volumes of 25 ml air are sucked through the system with the empty syringe to maintain the soil in an aerobic condition. A 1 ml aliquot of the alkali solution is taken for liquid scintillation counting.

(*6*) Experiment duration times of 1, 2, 4, 8, 16, 32 and—if necessary—64 days should be chosen for measurement. The test requires parallel determinations. The $^{14}CO_2$ radioactivity recovered is plotted versus time. This graph shows when to terminate the experiment. Incubation time is sufficient, when a total of 50 per cent $CO_2$ expressed as $^{14}C$ originally applied can be measured. Incubation should be stopped after reaching 64 days, whether or not this value is obtained.

(B) *Optional tests—(1) Estimation of evaporation.* If the volatility of a chemical is higher than $10^{-5}$ torr at 20 °C, it is recommended that a 3 cm diameter polyurethane foam plug be introduced into the arm E of the biometer flask. This plug absorbs the volatile parent compound as well as volatile organic degradation products but does not absorb $^{14}CO_2$. The plugs are extracted in a soxhlet extraction apparatus with an n-hexane/methanol mixture (1/4), and aliquots are taken for liquid scintillation counting.

(*2*) *Determination of soil-extractable and non-extractable residues.* (*i*) In cases of relatively persistent chemicals (50 per cent mineralisation in 10 days), further information concerning the soil-extractable radioactivity (parent compound plus degradation products) and soil bound residues is recommended.

(*ii*) For this purpose two further biometer flasks in addition to the four others must be prepared. At the point of 50 (or x-) percent mineralisation in the basic test, the soil in the two separate biometer flasks is extracted with 100 ml acetone (5 min ultrasonic treatment) followed by an extraction with methanol in the same manner. Aliquots of the combined extracts are taken for liquid scintillation counting. Other extract portions may be used—if necessary—for further identification studies.

(*iii*) Aliquots of the air dried soil are combusted to $^{14}CO_2$ and measured by liquid scintillation counting to determine the soil bound residues.

(c) *Data and reporting*—(1) *Treatment of results*—(i) *Basic test.* Radioactivity values for $^{14}CO_2$ (average of 4 parallel experiments) obtained from the alkali solution after 1, 2, 4, 8, 16, 32 and 64 days are expressed as the percentage of test chemical (radioactivity) initially applied and are plotted in a graph versus time. The time at which 50 per cent of the radioactivity is recovered as $^{14}CO_2$ is considered to be the "50% mineralisation" level. If this level has not been reached by the 64th day, the data at this time are taken and expressed as "x-percent-mineralisation".

(ii) *Evaporation test.* The radioactivity of vaporised (and trapped) original compound plus degradation products at the point of 50 (or x-) percent mineralisation is extracted, measured and interpreted as the percentage of volatilisation at the point of 50 (or x-) percent-mineralisation.

(iii) *Residue test.* Radioactivity values for extractable and non-extractable residues of the parent compound plus degradation products obtained after the extraction procedure of the soil at the point of 50 (or x-) percent mineralisation are given.

(2) *Test report.* The report of the degradability of a test chemical must include:

(i) Name of the test chemical, formula.

(ii) Amount applied, if not standard.

(iii) Exact characteristic data of the soil used.

(iv) Dates of the performance of the measurements.

(3) *Interpretation and evaluation of results.* The results are artificial because they are obtained with disturbed soil. However, since standardised soils are used, the test data are intercom-

parable and enable the experimentator to group relatively the chemicals tested within one scale for this property.

(d) *Literature references.* For additional background information on this test guideline the following references should be consulted:

(1) Bartha, R., and Pramer D., *Soil Science* 100: 68–70 (1965).

(2) Soil Survey Staff, U.S. Department of Agriculture, *Soil Taxonomy* Handbook No. 436, (Washington, DC, 1975).

(3) Butler, B.E., *Soil Classification for Soil Survey.* (Oxford, 1980) p. 129.

[50 FR 39512, Sept. 27, 1985]

## § 796.3480 Complex formation ability in water.

(a) *Introductory information.* (Polarographic Method)

(1) *Prerequisites.* (i) Suitable analytical method.

(ii) Water solubility.

(iii) Dissociation constant.

(2) *Guidance information.* Structural fomula.

(3) *Qualifying statement.* (i) This method is applicable only if the water solubility is higher than $10^{-5}$ M.

(ii) This method should be applied to pure substances.

(iii) This method is not applicable to the determination of mercury complexes.

(4) *Standard documents.* This Test Guideline is based on the consensus method of polarography.

(b) *Method*—(1) *Introduction, purpose, scope, relevance, application and limits of test.* (i)(A) The ability of a new chemical to form soluble metal complexes may increase the availability to food chains of metals that might otherwise become inaccessible, e.g. by incorporation in sediments. This is of outstanding importance in the case of cadmium, copper, cobalt, chromium, lead and zinc. The ability of a substance to form complexes with the above metals can be assessed by means of polarographic techniques which allow the determination of stability constants for some complexes.

(B) The polarographic method can be applied to substances with a water solubility greater than $10^{-5}$ M. Direct measurement of the shift in half-wave potential $E_{1/2}$ can be made, provided that reactions at the dropping-mercury electrode occur reversibly. With suitable modifications, irreversible cases can occasionally be dealt with. For example, the Gelling's method under paragraph (d)(6) of this section can be applied to calculate $E_{1/2}$ values for a reversible process from irreversible reduction data. For quasi-reversible systems the method proposed by Grabaric et al. under paragraph (d)(6) of this section may be conveniently used.

(C) Two or more ions may be determined successively, if their half-wave potentials differ by at least 0.4 V for univalent ions and 0.2 V for bivalent ions, provided that their concentrations are approximately equal.

(D) When a substance forms a series of complexes with a metal ion, the determination of stability constants for intermediate species is also possible, but generally not necessary for environmental assessment. With strong chelating agents care must be taken not to overload the system (i.e. that not all metal is in the complex form).

(ii) *Definitions and units.* (A) The potential E of the dropping-mercury electrode is given by the equation

$$E = E^{\circ} - \frac{RT}{nF} \ln \frac{[M_a]}{[M_s]}$$

Where:

$E^{\circ}$ is the standard electrode potential,
R is the gas constant,
T the absolute temperature,
n the number of electrons involved in the electrode process,
F the Faraday constant, and
$[M_a]$ and $[M_s]$ the concentrations of simple metals in the amalgam and at the electrode surface, respectively.

(B) The equation given above implies that the diffusion coefficients of the free metal ions and the complexed ones are equal.

(C) The stability constant for the complexation reaction

$$M^{n+} + pX^{b-} \rightleftharpoons MX_p(n-pb)+$$

is given by

$$K = \frac{[MX_p(n-pb)+]}{[M^{n+}][X^{b-}]^p}$$

Where:

p is the co-ordination number.

(D) The complex formation constant depends on the temperature and diffusion coefficient.

(E) By combination of the above equations, the following relation is obtained, which is valid for the reversible reduction of a complex species at 25 °C:

$$E_{1/2} = E_m - \frac{0.0591}{n} \log K [X] P$$

Where:

$E_{1/2}$ is the half-wave potential of the complex.

$E_m$ that of the metal ion in the absence of complexing agents, and

[X] the concentration of the test compound.

(F) SI unit of the half-wave potential is volt [V].

(iii) *Reference substances.* The following reference substances need not be employed in all cases when investigating a new substance. They are primarily provided so that calibration of the method may be performed from time to time and to offer the chance to compare the results when another method is applied.

(A) ethylenediaminetetraacetic acid (EDTA).

(B) nitrilotriacetic acid (NTA).

(C) thioglycolic acid.

(D) o-nitrophenol.

(iv) *Principle of the test method.* This method is based on the fact that the reduction potentials of metal ions are shifted, usually to more negative values, as a result of complex formation. A positive shift can also occur, but only if another metal ion is present in the solution that is capable of binding to excess complex-forming agent. The potential at the point on the polarographic wave where the current is equal to one half of the diffusion current is termed the "half-wave potential" ($E_{1/2}$).This parameter is a characteristic constant for each metal ion. In the presence of a complex-forming substance the extent of the $E_{1/2}$ shift depends on its concentration as well as on the stability of the complex. Measurements of $E_{1/2}$ as a function of substance concentrations permit the calculation of both the formulae and the stability constants of some metal complexes. It is assumed that the $E_{1/2}$ shift is entirely due to complex formation, with no contribution from liquid junction potential.

(v) *Quality criteria*—(A) *Repeatability.* Under normal conditions (particularly when working with metal ions in the concentration range $10^{-3}$ to $10^{-4}$ M the repeatability can be as good as ±2 per cent. The temperature coefficient is about 1.5–2% per °C. Precise measurements therefore require temperature control to within ±0.2 °C.

(B) *Sensitivity.* This depends upon the particular procedure adopted. Generally, metal ion concentrations down to $10^{-5}$ M can be determined.

(C) *Specificity.* The method can be applied to substances which are capable of forming stable aqueous solutions at concentrations greater than $10^{-5}$ M.

(D) *Possibility of standardization.* Yes.

(E) *Possibility of automation.* Not known.

(2) *Description of the test procedure*—(i) *Preparations.* Suitable polarographic apparatus has been fully described in the literature under paragraphs (d) (1) through (8) of this section.

(ii) *Test conditions.* (A) The mercury used in the dropping electrode should be at least redistilled commercial grade and should be filtered before use. Solutions to be tested need to be well-buffered under paragraph (d) (4) of this section. High-quality pure nitrogen should be used to de-oxygenate solutions for 10 to 15 minutes before determining the current-electrode potential curve.

(B) A minimum of four known concentrations of the chemical being tested should be investigated with a known concentration of metal ions. Solutions are most conveniently prepared directly within the polarographic cell by means of a precision burette. The chemical being tested should normally be present in at least a 25-fold excess over the metal ion concentrations so that its concentration at the electrode surface will be essentially the same as in the solution bulk. The current should be measured at applied potentials in the range −0.2 V to −1.0 V.

(C) In order to detect complexes which form slowly, it is necessary to allow the solutions to stand under a nitrogen atmosphere for a minimum period of 24 hours and, by re-examination of a sufficient number of samples, to ensure that complex formation was complete at the time of original measurements.

(D) The use of buffers and the use of surface-active substances to suppress polarographic maxima should be carefully considered, in order to avoid undesirable effects on the chemical reaction-controlled current as well as on the polarographic wave slope.

(iii) *Performance of the test.* The performance of the polarographic technique has been fully described in the literature paragraphs (d) (1) through (8) of this section. The test should be run at 25 °C (±0.2 °C).

(c) *Data and reporting*—(1) *Treatment of results.* The stability constant K can be easily determined by plotting $E_{1/2}$ versus log [X]P and extrapolating to [X]=1.0 M. If a straight line is obtained (whose slope is therefore 0.0591/n) the formation of a 1:1 complex is assumed. Smooth curves may be caused either by reaction irreversibility or by formation of two or more complex species in equilibrium with each other. In this last case it is necessary to apply the method by De Ford and Hume paragraph (d) (8) of this section to calculate stepwise formation constants.

(2) *Test report.* (i) The test report should list for each metal ion investigated the half-wave potential $E_{1/2}$, co-ordination number and overall stability constant.

(ii) In addition, the following should also be reported:

(A) Type of polarisable micro-electrode, the reference electrode and, in the case of the dropping mercury electrode, the flow rate in mg/sec and drop time.

(B) IR correction (if applied).

(C) Maxima suppressors (if used).

(D) Supporting electrolyte.

(E) Buffer.

(F) Temperature of measurement.

(G) Total ionic strength.

(H) Technique applied (pilot ion, standard addition, or other).

(I) Technical difficulties encountered.

(J) Estimate of accuracy.

(K) Polarographic technique employed (e.g., DC-, AC, polarography, single sweep polarography, radio frequency polarography or square-wave polarography).

(3) *Interpretation and evaluation of results.* (i) Stability constants determined for a new substance can be compared with literature values for standard substances (see Reference substances, above) and used therefore to evaluate the strength of its complexing ability.

(ii) The system is physically meaningful if (A) the value of the stability constant is positive and (B) the standard error is less than the constant (the t-test should be used as a criterion).

(iii) If data are not significant, it is necessary to use methods based on different physicochemical principles, such as spectrophotometry or nuclear magnetic resonance spectrometry.

(d) *Literature references.* For additional background information on this test guideline the following references should be consulted:

(1) Vogel, A.I., Basset, J. *Vogel's Textbook of Qualitative Inorganic Analysis.* Chapter XIX, 4th Ed. (Wiley: New York, 1978).

(2) Crow, D.R., Westwood, J.V., "The Study of Complexed Metal Ions by Polarographic Methods", *Quarterly Review 19:* 51 (1965).

(3) Irving, H., "The Stability of Metal Complexes and Their Measurement Polarographically," *Advances in Polarography Proceedings of the 2nd International Congress,* Ed. I.S. Langmuir (Pergamon Press, 1960).

(4) Perrin, D.D., Dempsey, B., *Buffer for pH and Metal Ion Controls.* (Chapman and Hall: London, 1974).

(5) "Stability Constants of Metal-ion Complexes," Part B, *Organic Ligands,* Compiled by D.D. Perrin, IUPAC Publication on Chemical Data Series, No. 22 (Pergamon Press, 1979)

(6) Grabaric, B., Tkalcec, M., Piljac, I., Filipovic, I., Simeon, V., "Numerical Evaluation of Complex Stability Constants from Polarographic Data for Quasi-Reversible Processes," *Analytica Chimica Acta 74*: 147, (1975).

(7) Piljac, I., Grabaric, B., Filipovic, I., "Improved Technique for Determination of Stability Constants by Polarographic Method," *Journal of Electroanalytical Chemistry Interfacial Electrochemistry, 42*:433 (1973).

(8) De Ford, D.D., Hume, D.N., "The Determination of Consecutive Formation Constants of Complex Ions from Polarographic Data," *American Chemical Society Journal, 73*:5321 (1951).

[50 FR 39514, Sept. 27, 1985]

## § 796.3500 Hydrolysis as a function of pH at 25 °C.

(a) *Introduction*—(1) *Background and purpose.* (i) Water is one of the most widely distributed substances in the environment. It covers a large portion of the earth's surface as oceans, rivers, and lakes. The soil also contains water, as does the atmosphere in the form of water vapor. As a result of this ubiquitousness, chemicals introduced into the environment almost always come into contact with aqueous media. Certain classes of these chemicals, upon such contact, can undergo hydrolysis, which is one of the most common reactions controlling chemical stability and is, therefore, one of the main chemical degradation paths of these substances in the environment.

(ii) Since hydrolysis can be such an important degradation path for certain classes of chemicals, it is necessary, in assessing the fate of these chemicals in the environment, to know whether, at what rate, and under what conditions a substance will hydrolyze. Some of these reactions can occur so rapidly that there may be greater concern about the products of the transformation than about the parent compounds. In other cases, a substance will be resistant to hydrolysis under typical environmental conditions, while, in still other instances, the substance may have an intermediate stability that can result in the necessity for an assessment of both the original compound and its transformation products. The importance of transformation of chemicals via hydrolysis in aqueous media in the environment can be determined quantitatively from data on hydrolysis rate constants. This hydrolysis Test Guideline represents a test to allow one to determine rates of hydrolysis at any pH of environmental concern at 25 °C.

(2) *Definitions and units.* (i) "Hydrolysis" is defined as the reaction of an organic chemical with water, such that one or more bonds are broken and the reaction products of the transformation incorporate the elements of water ($H_2O$).

(ii) "Elimination" is defined in this Test Guideline to be a reaction of an organic chemical (RX) in water in which the X group is lost. These reactions generally follow the same type of rate laws that hydrolysis reactions follow and, thus, are also covered in this Test Guideline.

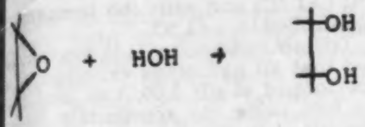
(iii) A "first-order reaction" is defined as a reaction in which the rate of disappearance of the chemical substance being tested is directly proportional to the concentration of the chemical substance and is not a function of the concentrations of any other substances present in the reaction mixture.

(iv) The "half-life" of a chemical is defined as the time required for the concentration of the chemical substance being tested to be reduced to one-half its initial value.

(v) "Hydrolysis" refers to a reaction of an organic chemical with water such that one or more bonds are broken and the reaction products incorporate the elements of water ($H_2O$). This type of transformation often results in the net exchange of a group X, on an organic chemical RX, for the OH group from water. This can be written as:

$$RX + HOH \rightarrow ROH + HX.$$

(A) Another result of hydrolysis can be the incorporation of both H and OH in a single product. An example of this is the hydrolysis of epoxides, which can be represented by



(B) The hydrolysis reaction can be catalyzed by acidic or basic species, including $OH^-$ and $H_3O^+$ ($H^+$). The promotion of the reaction by $H_3O^-$ or $OH^-$ is called specific acid or specific base catalysis, respectively, as contrasted with general acid or base catalysis encountered with other cationic or anionic species. Usually, the rate law for chemical RX can be written as:

*Equation 1*

$$-d[RX]/_{dt} = k_h[RX] = k_A[H^+][RX] + k_B[OH^-][RX] + k'_N[H_2O][RX],$$

where $K_A$, $k_B$ and $k'_N$ are the second-order rate constants for acid and base catalyzed and neutral water processes, respectively. In dilute solutions, such as are encountered in following this Test Guideline, water is present in great excess and its concentration is, thus, essentially constant during the course of the hydrolysis reaction. At fixed pH, the reaction, therefore, becomes pseudo first-order, and the rate constant ($k_h$) can be written as:

*Equation 2*

$$k_h = k_A[H^+] + k_B[OH^-] + k_N.$$

where $k_N$ is the first-order neutral water rate constant. Since this is a pseudo first-order process, the half-life is independent of the concentration and can be written as:

*Equation 3*

$$t_{1/2} = 0.693/k_h.$$

At constant pH, Equation 1 can be integrated to yield the first order rate expression

*Equation 4*

$$\log_{10} C = -(k_h t/_{2.303}) + \log_{10} C_0$$

where C is the concentration of the test chemical at time t and $C_0$ is the initial chemical concentration (t=0).

(C) At a given pH, Equation 2 under paragraph (a)(2)(v)(B) of this section contains three unknowns, $k_A$, $k_B$, and $k_N$. Therefore, three equations (i.e., measurements at three different pH's at a fixed temperature) are required if one wishes to solve for these quantities. Making suitable approximations for quantities that are negligible, the expressions for $k_A$, $k_B$, and $k_N$ using values of $k_h$ measured at pH 3, 7, and 11 are:

*Equation 5*

$$k_A = 10^3 [k_h(3) - k_h(7) + 10^{-4} k_h(11)]$$
$$k_B = 10^3 [k_h(11) - k_h(7) + 10^{-4} k_h(3)]$$
$$k_N = k_h(7) - 10^{-4} [k_h(3) + k_h(11)]$$

The calculated rate constants from equation 5 under this paragraph can be employed in equation 2 under paragraph (a)(2)(v)(B) of this section to calculate the hydrolysis rate of a chemical at any pH of environmental concern.

(D) The equations under paragraph (a)(2) of this section apply whether the test chemical has one or more hydrolyzable groups. In the latter case, the rate may be written as:

*Equation 6*

$$-d[RX]/_{dt} = [RX] + k_2 [RX] + \ldots\ldots + k_n [RX]$$
$$= (k_1 + k_2 + \ldots\ldots k_n)[RX] = k_h [RX].$$

Equation 6 applies to the hydrolysis rate of a molecule having n hydrolyzable groups, each of which follows first-order reaction kinetics. The measured $k_h$ is now the sum of the individual reaction rates and is the only rate constant required in this section.

(3) *Principle of the test method.* Procedures described in this section enable sponsors to obtain quantitative information on hydrolysis rates through a determination of hydrolysis rate constants and half-lives of chemicals at pH 3.00, 7.00, and 11.00 at 25 °C. The three measured rate constants are used to determine the acidic, basic, and neutral rate constants associated with a hydrolytic reaction. The latter constants can then be employed in determining the hydrolysis rates of chemicals at any pH of environmental concern at 25 °C.

(4) *Applicability and specificity.* There are several different common classes of organic chemicals that are subject to hydrolysis transformation,

including esters, amides, lactones, carbamates, organophosphates, and alkyl halides. Processes other than nucleophilic displacement by water can also take place. Among these are elimination reactions that exhibit behavior similar to hydrolysis and, therefore, are also covered in this section.

(b) *Test procedures*—(1) *Test conditions*—(i) *Special laboratory equipment.* (A) A thermostatic bath that can be maintained at a temperature of 25±1 °C.

(B) A pH meter that can resolve differences of 0.05 pH units or less.

(C) Stoppered volumetric flasks (no grease) or glass ampoules that can be sealed.

(ii) *Purity of water.* Reagent-grade water (e.g., water meeting ASTM Type IIA standards or an equivalent grade) shall be used to minimize biodegradation. ASTM Type IIA water is described in ASTM D 1193-77 (Reapproved 1983), "Standard Specification for Reagent Water." ASTM D 1193-77 (Reapproved 1983) is available for inspection at the Office of the Federal Register, Rm. 8301, 1100 L St., NW., Washington, DC. This incorporation by reference was approved by the Director of the Office of the Federal Register. This material is incorporated as it exists on the date of approval and a notice of any change in this material will be published in the FEDERAL REGISTER. Copies of the incorporated material may be obtained from the TSCA Public Docket Office (TS-793), Rm. NE-G004, Office of Toxic Substances, Environmental Protection Agency, 401 M St., SW., Washington, DC 20460, or from the American Society for Testing and Materials (ASTM), 1916 Race Street, Philadelphia, PA 19103.

(iii) *Sterilization.* All glassware shall be sterilized. Aseptic conditions shall be used in the preparation of all solutions and in carrying out all hydrolysis experiments to eliminate or minimize biodegradation. Glassware can be sterilized in an autoclave or by any other suitable method.

(iv) *Precautions for volatility.* If the chemical is volatile the reaction vessels shall be almost completely filled and sealed.

(v) *Temperature controls.* All hydrolysis reactions shall be carried out at 25 °C (±1 °C) and with the temperature controlled to ±0.1 °C.

(vi) *pH conditions.* It is recommended that all hydrolysis experiments be performed at pH 3.00, 7.00, and 11.00 ± 0.05 using the appropriate buffers described in paragraph (b)(2)(i)(A) of this section.

(vii) *Concentration of solutions of chemical substances.* The concentration of the test chesical shall be less than one-half the chemical's solubility in water but not greater than $10^{-3}$M.

(viii) *Effect of acidic and basic groups.* Complications can arise upon measuring the rate of hydrolysis of chemicals that reversibly ionize or are protonated in the pH range 3.00 to 11.00. Therefore, for these chemicals, it is recommended that these hydrolysis tests be performed at pH 5.00, 7.00, and 900±0.05 using the appropriate buffers described in paragraphs (b)(2)(i) (A) and (B) of this section. If a test chemical reversibly ionizes or protonates in the pH range 5.00 to 9.00, then it is recommended that additional hydrolysis tests should be carried out at pH 6.00 and 8.00±0.05 using the buffers described in paragraph (b)(2)(i)(B) of this section.

(ix) *Buffer catalysis.* For certain chemicals, buffers may catalyze the hydrolysis reaction. If this is suspected, hydrolysis rate determination shall be carried out with the appropriate buffers and the same experiments repeated at buffer concentrations lowered by at least a factor of five. If the hydrolysis reaction produces a change of greater than 0.05 pH units in the lower concentration buffers at the end of the measurement time, the test chemical concentrations also shall be lowered by at least a factor of five. Alternatively, test chemical concentrations and buffer concentrations may both be lowered simultaneously by a factor of five. A sufficient criterion for minimization of buffer catalysis is an observed equality in the hydrolysis rate constant for two different solutions differing in buffer or test chemical concentration by a factor of five.

(x) *Photosensitive chemicals.* The solution absorption spectrum can be employed to determine whether a particular chemical is potentially subject to photolytic transformation upon exposure to light. For chemicals that absorb light of wavelengths greater than 290 nm, the hydrolysis experiment shall be carried out in the dark, under amber or red safelights, in amber or red glassware, or employing other suitable methods for preventing photolysis. The absorption spectrum of the chemical in aqueous solution can be measured under § 796.1050.

(xi) *Chemical analysis of solutions.* In determining the concentrations of the test chemicals in solution, any suitable analytical method may be employed, although methods which are specific for the compound to be tested are preferred. Chromatographic methods are recommended because of their compound specificity in analyzing the parent chemical without interferences from impurities. Whenever practicable, the chosen analytical method should have a precision within ±5 percent.

(2) *Preparation*—(i) *Reagents and solutions*—(A) *Buffer solutions.* Prepare buffer solutions using reagent-grade chemicals and reagent-grade water as follows:

(*1*) pH 3.00: use 250 mL of 0.100M potassium hydrogen phthalate; 111 mL of 0.100M hydrochloric acid; and adjust volume to 500 mL with reagent-grade water.

(*2*) pH 7.00: use 250 mL of 0.100M potassium dihydrogen phosphate; 145 mL of 0.100M sodium hydroxide; and adjust volume to 500 mL with reagent-grade water.

(*3*) pH 11.00: use 250 mL of 0.0500M sodium bicarbonate; 113 mL of 0.100M sodium hydroxide; and adjust volume to 500 mL with reagent-grade water.

(B) *Additional buffer solutions.* For chemicals that ionize or are protonated as discussed in paragraph (b)(1)(viii) of this section, prepare buffers using reagent-grade water and reagent-grade chemicals as follows:

(*1*) pH 5.00: use 250 mL of 0.100M potassium hydrogen phthalate; 113 mL of 0.100M sodium hydroxide; and adjust volume to 500 mL with reagent-grade water.

(*2*) pH 6.00: use 250 mL of 0.100M potassium dihydrogen phosphate; 28 mL of 0.100M sodium hydroxide; and adjust volume to 500 mL with reagent-grade water.

(*3*) pH 8.00: use 250 mL of 0.100M potassium dihydrogen phosphate; 234 mL of 0.100M sodium hydroxide; and adjust volume to 500 mL with reagent-grade water.

(*4*) pH 9.00: use 250 mL of 0.0250M borax ($Na_2B_4O_7$); 23 mL of 0.100M hydrochloric aid; and adjust volume to 500 mL with reagent-grade water.

(C) *Adjustment of buffer concentrations.* (*1*) The concentrations of all the above buffer solutions are the maximum concentration to be employed in carrying out hydrolysis measurements. If the initial concentration of the test chemical is less than $10^{-3}$M, the buffer concentration shall be lowered by a corresponding amount; e.g., if the initial test chemical concentration is $10^{-4}$M, the concentration of the above buffers shall be reduced by a factor of 10. In addition, for those reactions in which an acid or base is not a reaction product, the minimum buffer concentration necessary for maintaining the pH within +0.05 units shall be employed.

(*2*) Check the pH of all buffer solutions with a pH meter at 25 °C and adjust the pH to the proper value, if necessary.

(D) *Preparation of test solution.* (*1*) If the test chemical is readily soluble in water, prepare an aqueous solution of the chemical in the appropriate buffer and determine the concentration of the chemical. Alternatively, a solution of the chemical in water may be prepared and added to an appropriate buffer solution and the concentration of the chemical then determined. In the latter case, the aliquot shall be small enough so that the concentration of the buffer in the final solution and the pH of the solution remain essentially unchanged. Do not employ heat in dissolving the chemical. The final concentration shall not be greater than one-half the chemical's solubility in water and not greater than $10^{-3}$M.

(*2*) If the test chemical is too insoluble in pure water to permit reasonable handling and analytical procedures, it is recommended that the chemical be dissolved in reagent-grade acetonitrile and buffer solution and then added to an aliquot of the acetonitrile solution. Do not employ heat to dissolve the chemical in acetonitrile. The final concentration of the test chemical shall not be greater than one-half the chemical's solubility in water and not greater than $10^{-3}$M. In addition, the final concentration of the acetonitrile shall be one volume percent or less.

(3) *Performance of the test.* Carry out all hydrolysis experiments by employing one of the procedures described in this paragraph. Prepare the test solutions as described in paragraph (b)(2)(i) of this section at pH 3.00, 7.00, and 11.00±0.05, and determine the initial test chemical concentration ($C_o$) in triplicate. Analyze each reaction mixture in triplicate at regular intervals, employing one of the following procedures:

(i) *Procedure 1.* Analyze each test solution at regular intervals to provide a minimum of six measurements with the extent of hydrolysis between 20 to 70 percent. Rates should be rapid enough so that 60 to 70 percent of the chemical is hydrolyzed in 672 hours.

(ii) *Procedure 2.* If the reaction is too slow to conveniently follow hydrolysis to high conversion in 672 hours but still rapid enough to attain at least 20 percent conversion, take 15 to 20 time points at regular intervals after 10 percent conversion is attained.

(iii) *Procedure 3.* (A) If chemical hydrolysis is less than 20 percent after 672 hours, determine the concentration (C) after this time period.

(B) If the pH at the end of concentration measurements employing any of the above three procedures has changed by more than 0.05 units from the initial pH, repeat the experiment using a solution having a test chemical concentration lowered sufficiently to keep the pH variation within 0.05 pH units.

(iv) *Analytical methodology.* Select an analytical method that is most applicable to the analysis of the specific chemical being tested under paragraph (b)(1)(xi) of this section.

(c) *Data and reporting*—(1) *Treatment of results.* (i) If Procedure 1 or 2 were employed in making concentration measurements, use a linear regression analysis with Equation 4 under paragraph (a)(2)(v)(B) of this section to calculate $k_h$ at 25 °C for each pH employed in the hydrolysis experiments. Calculate the coefficient of determination ($R^2$) for each rate constant. Use Equation 3 under paragraph (a)(2)(v)(B) of this section to calculate the hydrolysis half-life using $k_h$.

(ii) If Procedure 3 was employed in making rate measurements, use the mean initial concentration ($C_o$) and the mean concentration of chemical (C) in Equation 4 under paragraph (a)(2)(v)(B) of this section to calculate $k_h$ for each pH used in the experiments. Calculate the hydrolysis half-life using $k_h$ in Equation 3 under paragraph (a)(2)(v)(B) of this section.

(iii) For each set of three concentration replicates, calculate the mean value of C and the standard deviation.

(iv) For test chemicals that are not ionized or protonated between pH 3 and 11, calculate $k_A$, $k_B$, and $k_N$ using Equation 5.

(2) *Specific analytical and recovery procedures.* (i) Provide a detailed description or reference for the analytical procedure used, including the calibration data and precision.

(ii) If extraction methods were used to separate the solute from the aqueous solution, provide a description of the extraction method as well as the recovery data.

(3) *Test data report.* (i) For Procedures 1 and 2, report $k_h$, the hydrolysis half-life ($t_{1/2}$), and the coefficient of determination ($R^2$) for each pH employed in the rate measurements. In addition, report the individual values, the mean value, and the standard deviation for each set of replicate concentration measurements. Finally, report $k_A$, $k_B$, and $k_N$.

(ii) For Procedure 3, report $k_h$ and the half-life for each pH employed in the rate measurements. In addition, report the individual values, the mean value, and the standard deviation for each set of replicate concentration measurements. Finally, report $k_A$, $k_B$, and $k_N$.

(iii) If, after 672 hours, the concentration (C) is the same as the initial concentration ($C_o$) within experimental error, then $k_h$ cannot be calculated and the chemical can be reported as being persistent with respect to hydrolysis.

[50 FR 39252, Sept. 27, 1985, as amended at 53 FR 10391, Mar. 31, 1988; 53 FR 12526, Apr. 15, 1988; 53 FR 22323, June 15, 1988]

### § 796.3700 Photolysis in aqueous solution in sunlight.

(a) *Introduction*—(1) *Background and purpose.* Numerous chemicals enter natural aquatic systems from a variety of sources. For example, chemical wastes are discharged directly into natural water bodies, and chemicals leach into natural water bodies from landfills. Pesticides are applied directly into water bodies, and are applied to soils and vegetation, and subsequently leach into water bodies. Pollutants present in aqueous media can undergo photochemical transformation in the environment (i.e., in sunlight by direct photolysis or by sensitized photolysis). As a result, there is considerable interest in photolysis in solution, especially the photolysis of pesticides. However, most of these studies have been qualitative in nature and involved the identification of photolysis products. Quantitative data in the form of rate constants and half-lives are needed to determine the importance of photochemical transformation of pollutants in aqueous media. This test method describes a two-tiered screening level approach for determining direct photolysis rate constants and half-lives of chemicals in water in sunlight.

(2) *Definitions and units.* (i) "Radiant energy," or radiation, is defined as the energy traveling as a wave unaccompanied by transfer of matter. Examples include x-rays, visible light, ultraviolet light, radio waves, etc.

(ii) "Absorbance ($A_\lambda$)" is defined as the logarithm to the base 10 of the ratio of the initial intensity ($I_o$) of a beam of radiant energy to the intensity (I) of the same beam after passage through a sample at a fixed wavelength λ. Thus, $A_\lambda = \log_{10}(I_o/I)$.

(iii) The "Beer-Lambert law" states that the absorbance of a solution of a given chemical species, at a fixed wavelength, is proportional to the thickness of the solution (l), or the light pathlength, and the concentration of the absorbing species (C).

(iv) "Molar absorptivity ($\epsilon_\lambda$)" is defined as the proportionality constant in the Beer-Lambert law when the concentration is given in terms of moles per liter (i.e., molar concentration). Thus, $A_\lambda = \epsilon_\lambda Cl$, where $A_\lambda$ and $\epsilon_\lambda$ represent the absorbance and molar absorptivity at wavelength λ and *l* and C are defined in (3). The units of $\epsilon_\lambda$ are $\text{molar}^{-1}\ \text{cm}^{-1}$. Numerical values of molar absorptivity depend upon the nature of the absorbing species.

(v) A "first-order reaction" is defined as a reaction in which the rate of disappearance of a chemical is directly proportional to the concentration of the chemical and is not a function of the concentration of any other chemical present in the reaction mixture.

(vi) The "half-life ($t_{1/2}$)" of a chemical is defined as the time required for the concentration of the chemical being tested to be reduced to one-half its initial value.

(vii) The "sunlight direct aqueous photolysis rate constant ($k_{pE}$)" is the first-order rate constant in the units of $\text{day}^{-1}$ and is a measure of the rate of disappearance of a chemical dissolved in a water body in sunlight.

(viii) The "solar irradiance in water ($L_\lambda$)" is related to the sunlight intensity in water and is proportional to the average light flux (in the units of $10^{-3}$ einsteins $\text{cm}^{-2}\ \text{day}^{-1}$) that is available to cause photoreaction in a wavelength interval centered at λ over a 24-hour day at a specific latitude and season date.

(ix) "The Grotthus-Draper law," the first law of photochemistry, states that only light which is absorbed can be effective in producing a chemical transformation.

(x) The "Stark-Einstein law," the second law of photochemistry, states that only one molecule is activated to an excited state per photon or quantum of light absorbed.

(xi) The "reaction quantum yield ($\phi_\lambda$)" for an excited-state process is defined as the fraction of absorbed light that results in photoreaction at a fixed wavelength λ. It is the ratio of the number of molecules that photoreact to the number of quanta of light absorbed or the ratio of the number of moles that photoreact to the number of einsteins of light absorbed at a fixed wavelength λ.

(xii) "Direct photolysis" is defined as the direct absorption of light by a chemical followed by a reaction which transforms the parent chemical into one or more products.

A glossary of symbols can be found under paragraph (c)(5) of this section.

(3) *Principle of the test method.* (i) This test method is based on the principles developed by Zepp and Cline (1977) under paragraph (d)(8) of this section, Zepp (1978) under paragraph (d)(11) of this section, Mill et al. (1981, 1982) under paragraphs (d) (4), (5), and (6) of this section, and Dulin and Mill (1982) under paragraph (d)(2) of this section.

(ii) Zepp and Cline (1977), under paragraph (d)(8) of this section published a paper on the rates of direct photolysis in aquatic environments. The rates of all photochemical processes in a water body are affected by solar spectral irradiance at the water surface, radiative transfer from air to water, and the transmission of sunlight in the water body. It has been shown that for photolysis of a chemical in an optically thin aqueous solution, the kinetics of direct photolysis can be described by the following equations:

*Equation 1*

$$\ln (C_o/C_t)=k_{pE}t$$

*Equation 2*

$$t_{1/2E}=0.693/k_{pE}$$

*Equation 3*

$$k_{pE}=\phi_E k_a,$$

where $\phi_E$ is the reaction quantum yield of the chemical in dilute solution and is independent of the wavelength, $k_a=\Sigma k_{a\lambda}$, the sum of $k_{a\lambda}$ values of all wavelengths of sunlight that are absorbed by the chemical (i.e., the light absorption rate constant), t is the time, $C_o$ and $C_t$ are the concentrations of chemical at t=0 and t, and $t_{1/2E}$ represents the half-life. The term $k_{pE}$ represents the first-order photolysis rate constant for a water body in sunlight in the units of reciprocal time.

(iii) Furthermore, under the same conditions cited above, the first-order direct photolysis rate constant, $k_{pE}$, is given by the equation—

*Equation 4*

$$k_{pE}=\phi_E\Sigma\epsilon_\lambda L_\lambda,$$

where $\phi_E$ is the reaction quantum yield, $\epsilon\lambda$ is the molar absorptivity in the units molar$^{-1}$ cm$^{-1}$, $L_\lambda$ is the solar irradiance in water in the units of $10^{-3}$ einsteins cm$^{-2}$ day$^{-1}$ [Mill et al. (1982) under paragraph (d)(5) of this section], and the summation is taken over the range $\lambda$=290 to 800 nm. $L_\lambda$ is the solar irradiance at shallow depths for a water body under clear sky conditions and is a function of latitude and season of the year.

(iv) The method of Zepp and Cline (1977) under paragraph (d)(8) of this section and the method of Mill et al. (1982) under paragraph (d)(5) of this section are applicable to sunlight incident on a water surface such as natural water body. However, the method developed in this guideline measures rate constants in tubes (e.g., 13×100 mm) and the rate is faster in tubes. This is discussed in more detail in paragraph (b)(2)(i)(J) of this section. Thus, equations 1 and 2 have to be modified to take this into account. For simplicity, the following nomenclature is used. For water bodies, the rate constant is designated as $k_{pE}$ with the subscript E designating rates in the environment in water bodies. For tubes, the rate constant is designated as $k_p$. The corresponding half-lives for water bodies and tubes are $t_{1/2E}$ and $t_{1/2}$, respectively. Thus, for tubes, equations 1 and 2 can be written as:

*Equation 5*

$$\ln(C_o/C_t)=k_pt$$

*Equation 6*

$$t½=\frac{0.693}{k_p}.$$

(v) A simple first-tier screening test has been developed using Equation 4 under paragraph (a)(3)(iii) of this section. As an approximation, it is assumed that the reaction quantum yield $\phi_E$ is equal to one, the maximum value. As a result, the upper limit for the direct photolysis sunlight rate constant in aqueous solution is obtained and Equation 4 under paragraph (a)(3)(iii) of this section becomes

*Equation 7*

$(k_{pE})_{max} = \Sigma \epsilon_\lambda L_\lambda$.

Using equation 7 in equation 2 under paragraph (a)(3)(ii) of this section, the lower limit for the half-life is then given by

*Equation 8*

$$(t_{1/2E})_{min} = \frac{0.693}{(k_{pE})_{max}}.$$

The molar absorptivity can be determined experimentally by the method outlined in paragraph (b)(1) of this section and values of $L_\lambda$ are given in Tables 3 to 6 as a function of latitude and season of the year under paragraph (c)(3) of this section. These data can then be used in equation 7 to calculate $(k_{pE})_{max}$. Finally, $(k_{pE})_{max}$ can then be substituted in Equation 8 to calculate $(t_{1/2E})_{min}$.

(vi) In a second-tier test method, an aqueous photolysis screening test has been developed to determine rate constants and half-lives in the presence of sunlight using Equations 1, 2, 4, 5, and 6 [Mill et al. (1981, 1982, under paragraphs (d) (4), (5) and (6) of this section, and Dulin and Mill (1982) under paragraph (d)(2) of this section]. The second-tier test method is divided into two phases. In phase one, the test chemical is photolyzed in sunlight in order to obtain an approximate rate constant, $k^c_p$. This method only gives an approximate rate constant since it fails to measure sunlight intensities incident on the sample during photolysis.

(vii) In phase two, a standard p-nitroacetophenone-pyridine actinometer (PNAP/PYR) is used to measure sunlight intensities incident on the sample during photolysis [Mill et al. (1982) under paragraph (d)(6) of this section and Dulin and Mill (1982) under paragraph (d)(2) of this section]. The rate constant for this actinometer, $k^a_p$, can be adjusted to match the approximate rate constant of the test chemical by adjusting the concentration of pyridine. Since the rate constant is a function of the reaction quantum yield of the actinometer, the rate constant can be adjusted according to the equation

*Equation 9*

$\phi^a_E = 0.0169[PYR]$,

where [PYR] is the molar concentration of pyridine for a p-nitroacetophenone (PNAP) concentration of 1.00 × $10^{-5}$ M. The reaction quantum yield for the test chemical, $\phi^c_E$, is given by

*Equation 10*

$$\phi^c_E = \frac{k^c_p}{k^a_p} \frac{\Sigma \phi^a_\lambda L_\lambda}{\Sigma \phi^c_\lambda L_\lambda} \phi^a_E .$$

The reaction quantum yield of the test chemical, $\phi^c_E$, can be determined in the following way. By measuring the concentration of test chemical and actinometer (PNAP) as a function of time t in sunlight, the ratio of rate constants, $(k^c_p/k^a_p)$, can be determined using Equation 5 under paragraph (b)(2)(i)(H) of this section. The reaction quantum yield $\phi^a_E$ can be determined from Equation 9 at the molar concentration of pyridine used in the standard actinometer. The term $\Sigma \epsilon^a_\lambda L_\lambda$ for the actinometer has been tabulated as a function of latitude and season of the year in Table 2 under paragraph (c)(3) of this section. The term $\Sigma \epsilon^c_\lambda L_\lambda$ for the test chemical can be obtained from the experimentally measured molar absorptivities under paragraph (b)(1) of this section and the values of $L_\lambda$ listed in Tables 3 to 6, as a function of latitude and season of the year under paragraph (c)(3) of this section.

(viii) With the values of $\phi^c_E$, $\epsilon^c_\lambda$, and the appropriate $L_\lambda$ values, $k_{pE}$ for the test chemical can be calculated as a function of latitude and season of the year in the United States using Equation 4 under paragraph (a)(3)(iii) of this section. The corresponding half-life can be calculated using $k_{pE}$ in Equation 2 under paragraph (a)(3)(ii) of this section.

(4) *Applicability and specificity.* (i) This test method is applicable to all chemicals which have UV-visible absorption maxima in the range of 290 to 800 nm. Some chemicals have absorption maxima significantly below 290

nm and consequently cannot undergo direct photolysis in sunlight (e.g., chemicals such as alkanes, alkenes, alkynes, saturated alcohols, and saturated acids). This is a direct consequence of the Grotthus-Draper law of photochemistry. Some chemicals have absorption maxima significantly below 290 nm but have measurable absorption tails above the baseline in their absorption spectrum at wavelengths greater than 290 nm. Photolysis experiments should be carried out for these chemicals.

(ii) These test methods are only applicable to pure chemicals and not to the technical grade.

(iii) The first-tier screening test can be employed to estimate $(k_{dE})_{max}$ and $(t_{1/2E})_{min}$. If these data indicate that aqueous photolysis is an important process relative to other transformation processes (e.g., biodegradation, hydrolysis, oxidation, etc.), then it is recommended that the second-tier photolysis tests be carried out to determine environmentally relevant rate constants and half-lives in sunlight. The data obtained from this test can be used to determine $k_{dE}$ for the test chemical as a function of latitude and season of the year anywhere in the United States. These rate constants are in a form suitable for preliminary mathematical modeling for environmental fate of a test chemical.

(iv) The second-tier screening test is applicable to the direct photolysis of chemicals in a homogeneous dilute solution with absorbance less than 0.05 in the reaction cell at all wavelengths greater than 290 nm and at shallow depths (less than 0.5 m). These results are applicable to direct sunlight photolysis for water bodies and clear sky conditions. In addition, these experiments are limited to the direct photolysis of chemicals in air-saturated pure water.

(v) This screening test has been designed to determine the molar absorptivity of a test chemical, $\epsilon_{\lambda}^{c}$, and its reaction quantum yield, $\phi^{c}_{E}$. These parameters can be used to determine environmentally relevant rate constants at low absorbance and shallow depths in pure water as a function of latitude and season of the year. Tables of solar irradiance (Tables 3 to 6) under paragraph (c)(3) of this section have been included in this test method to carry out all the calculations. However, the method is really very general and can be extended to determine the rates of photolysis over a range of other environmental conditions using a computer program. Zepp and Cline (1977) under paragraph (d)(8) of this section have written a computer program to calculate the rates of photolysis as a function of depth in water, as a function of the attenuation coefficient of the water ($\alpha_{\lambda}$) for natural water bodies, the average ozone layer thickness that pertains to the seasons and location of interest, and as a function of latitude and season of the year. This program has been recently updated with the best available solar irradiance data and is called the GC SOLAR program. The GC SOLAR computer program is available on request as referenced under paragraph (d)(10) of this section.

(b) *Test procedures*—(1) *Tier 1 Test: UV-visible absorption spectra-estimation of aqueous photolysis maximum rate constant and minimum half-life in sunlight.* The UV-visible absorption spectra in aqueous solution can be determined by the methods described in § 796.1050. It is recommended that the following additional procedures be followed:

(i) For chemicals which ionize or protonate (e.g., carboxylic acids, phenols, amines), carry out uv-visible absorption studies at pHs at least two orders or magnitude above the $pK_a$ and at least two orders of magnitude below the $pK_a$. Prepare buffer solutions at 25 °C using reagent grade chemicals and distilled water as follows:

pHs in the range 3–6[1]: $NaH_2PO_4$,HCl;

pHs in the range 6–8[1]: $KH_2PO_4$, NaOH;

pHs in the range >8: Prepare buffers as described in the Handbook of Chemistry and Physics.

Check the pH of all the buffer solutions with a pH meter at 25 °C and adjust to the proper pH, if necessary. These buffer solutions can then be added to the test chemical solution until the desired pH is obtained. If these buffers are inadequate, then adjust the pH of the test chemical solution with 1 M HCl or NaOH at 25 °C.

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[1] Use the minimum concentration of buffers to attain the desired pH.

(ii) (A) Measure the absorbance, $A_\lambda$, as a function of wavelength in the range of 290 to 800 nm in duplicate. If applicable, measure $A_\lambda$ at each experimental pH. Record, in duplicate, the baseline when both the sample and reference cells are filled with blank solutions. These data will be used to calculate the molar absorptivities for the appropriate wavelength intervals and wavelength centers in Table 1 under paragraph (c)(3) of this section, where the test chemical absorbs light. The wavelength center is defined as the midpoint of the interval range.

(B) It must be emphasized that the molar absorptivities of the test chemical must be carefully determined, especially in the tails of the absorption bands at $\lambda$ >290 nm. Large errors will be encountered in calculating photolysis rate constants and half-lives if these measurements are not carefully carried out.

(2) *Tier 2 Test: Aqueous Photolysis in Sunlight*—(i) *Test conditions*—(A) *Special laboratory equipment.* It is recommended that quartz tubes be used for the photolysis of chemicals with appreciable absorption at wavelengths below 340 nm. Chemicals that absorb appreciably at wavelengths greater than 340 nm may be tested in borosilicate tubes. Thin-walled borosilicate or quartz tubes are recommended. Disposable culture tubes (13 x 100 mm) with Teflon-lined screw caps or quartz tubes with quartz or borosilicate stoppers, Teflon-lined, may be used as reaction vessels. Tubes of 11 mm i.d. are recommended. For some chemicals, it may be difficult to determine the concentration of the test chemical in reaction tubes of small volume. For these chemicals, larger volume reaction vessels are recommended provided that the cell walls are thin and the pathlength of radiation through the vessel is less than 0.5 meter.

(B) *Purity of water.* Reagent grade water, e.g., water meeting ASTM Type II A standards, or an equivalent grade, is highly recommended to minimize biodegradation. ASTM Type II A water is described in ASTM D 1193-77 (Reapproved 1983), "Standard Specification for Reagent Water." Air-saturated water can be easily prepared by allowing the water to equilibrate in a vessel plugged with sterile cotton. ASTM D 1193-77 (Reapproved 1983) is available for inspection at the Office of the Federal Register, Rm. 8401, 1100 L St., NW., Washington, DC. This incorporation by reference was approved by the Director of the Office of the Federal Register. This material is incorporated as it exists on the date of approval and a notice of any change in this material will be published in the FEDERAL REGISTER. Copies of the incorporated material may be obtained from the TSCA Public Docket Office (TS-793), Rm. NE-G004, Office of Toxic Substances, Environmental Protection Agency, 401 M St., SW., Washington, DC 20460, or from the American Society for Testing and Materials (ASTM), 1916 Race Street, Philadelphia, PA 19103.

(C) *Sterilization.* It is extremely important to sterilize all glassware and to use aseptic conditions in the preparation of all solutions and in carrying out all photolysis experiments to eliminate or minimize biodegradation. Glassware can be sterilized in an autoclave or by any other suitable nonchemical method.

(D) *pH effects.* It is recommended that all photolysis experiments be carried out at pHs at least two orders of magnitude above the $pK_a$ and at least two orders of magnitude below the $pK_a$ for any chemical which ionizes or protonates (e.g., carboxylic acids, phenols, and amines). Buffers described in paragraph (b)(2)(ii)(B) of this section should be used.

(E) *Volatile chemical substances.* Special care should be taken when testing a volatile chemical so that the chemical substance is not lost due to volatilization during the course of the photolysis experiment. Thus, it is important to effectively seal the reaction vessels. Disposable culture tubes with Teflon-lined screw caps or quartz tubes with quartz or borosilicate stoppers, Teflon-lined, are recommended. Volatile compounds can be conveniently studied in culture tubes equipped with Mininert® valves. Samples can be introduced into or removed from the tubes through the septum in these valves with no loss of substrate. As an alternative, the tubes can be sealed with a torch. In addition, the reaction vessels should be as completely filled as is possible to prevent volatilization to any air space.

(F) *Control solution.* It is extremely important to take certain precautions to prevent loss of chemical from the reaction vessels by processes other than photolysis. For example, biodegradation and volatilization can be eliminated or minimized by use of sterile conditions and minimal airspace in sealed vessels. Hydrolysis is a process which cannot be minimized by such techniques. Thus, control vessels containing test substances which are not exposed to sunlight are required. In this way, the loss of test chemical for processes other than photolysis may be determined and eliminated. For simplicity, if the loss of chemical in the control is small (i.e., approximately 10 percent or less), one can calculate a first-order loss, $k_{loss}$, and subtract it from $(k_p)_{obs}$. to give the corrected direct photolysis rate constant $k_p$. If hydrolysis is found to be significant (i.e., greater than 10 percent), hydrolysis studies should be carried out first under § 796.3500.

(G) *Absorption spectrum as a criterion for performing the aqueous photolysis test.* This aqueous photolysis screening test is applicable to all chemicals which have UV-visible absorption maxima in the range of 290 to 800 nm. Some chemicals have absorption maxima significantly below 290 nm but have measurable absorption tails above the baseline in their absorption spectrum at wavelengths greater than 290 nm. Photolysis experiments should be carried out for these chemicals. The absorption spectrum of the chemical in aqueous solution can be measured by § 796.1050.

(H) *Sunlight actinometer.* (*1*) In order to quantify the rate of photolysis more precisely, it is necessary to measure the sunlight intensity incident on the sample during photolysis. A standard p-nitroacetophenone-pyridine actinometer (PNAP/PYR) has been developed [Mill et al. (1981, 1982) under paragraphs (d) (4) and (6) of this section; Dulin and Mill (1982) under paragraph (d)(2) of this section] to measure the sunlight intensity incident on the sample during photolysis and this actinometer has been incorporated in this section. According to Equation 4 under paragraph (a)(3)(iii) of this section, the rate constant is a function of the reaction quantum yield. Furthermore, the reaction quantum yield can be adjusted by varying the molar concentration of the pyridine according to Equation 9 under paragraph (a)(3)(vii) of this section. Hence, by varying the pyridine concentration, the actinometer photolysis rate constant can be adjusted so that the half-life can range from several hours to several weeks. The initial concentration of PNAP is set at 1.00 x $10^{-5}$ M.

(*2*) Using the test chemical photolysis rate constant, $k_p^c$, determined in Tier 2, Phase 1, and the variable $k_a^a(=\Sigma\epsilon^a{}_\lambda L_\lambda)$, listed in Table 2 under paragraph (c)(3) of this section the molar concentration needed to adjust the rate of disappearance of PNAP in PNAP/PYR to match the rate of disappearance of the test chemical is given by

*Equation 11*

$$[PYR]=26.9\ (k_p^c/k_a^a).$$

(*3*) Experiments are carried out by simultaneously photolyzing the test chemical and actinometer solutions. The concentrations of test chemical and actinometer are measured periodically as a function of time. These data are then used to determine the ratio of the rate constants, $k_p^c/k_p^a$, using linear regression analysis on the following equation:

*Equation 12*

$$\ln(C_o/C_t)^c=(k_p^c k_p^a)\ \ln(C_o/C_t)^a,$$

with ln $(C_o/C_t)^a$ as the independent variable and $\ln(C_o/C_t)^c$ as the dependent variable. The slope of the best straight line is the ratio of the rate constants, $k_p^c/k_p^a$.

(I) *Solar irradiance data.* In order to calculate the reaction quantum yield of the test chemical, $\phi_E^c$, and then calculate $k_{pE}^c$ and $t_{1/2E}$, it is $t_{1/2E}$ necessary to use the solar irradiance parameter $L_\lambda$. $L_\lambda$ values are proportional to the average light flux that is available to cause photolysis in a wavelength interval centered at λ over a 24-hour day at a specific latitude and season date. The $I_\lambda$ values are defined by the angle of declination of the sun at −20° for winter, −10° for fall, +10° for spring, and +20° for summer. The actual dates for 1982 that correspond to these angles of declination are January 21, April 16, July 24, and October 20, for winter, spring, summer, and fall, respectively [AA (1982) under paragraph (d)(1) of this section]. The $I_\lambda$ values for these season dates are listed in Tables 3 to 6 under paragraph (c)(3) of this section as a function of latitude and are applicable to clear sky conditions, water bodies, shallow depths, and for chemicals whose absorbance is less than 0.05 in pure water [Mill et al. (1984) under paragraph (d)(7) of this section].

(J) *Geometry of the reaction vessel.* The method of Zepp and Cline (1977) under paragraph (d)(8) of this section and the method of Mill et al., (1982) under paragraph (d)(5) of this section are applicable to sunlight incident on a water surface such as a natural water body while the method developed in this test method measures rate constants ($k_p$) in tubes (e.g., 13 × 100 mm). However, rates in tubes are faster than in water bodies and it has been experimentally observed [Mill et al. (1982) under paragraph (d)(6) of this section] that

*Equation 13*

$$k_p = 2.2k_{pE}.$$

Because tubes are the simplest and easiest reaction vessels to use, this test method recommends the use of tubes as reaction vessels and the method has been modified to take into account the increased rate in tubes (equation 13).

(K) *Chemical analysis of solution.* (*1*) In determining the concentration of the chemical in solution, an analytical method should be selected which is most applicable to the analysis of the specific chemical substance. Chromatographic methods are generally recommended because of their chemical specificity in analyzing the parent chemical substance without interference from impurities. Whenever practicable the chosen analytical method should have a precision of ±5 percent or better.

(*2*) The p-nitroacetophenone in the chemical actinometer solution is conveniently analyzed by high-pressure liquid chromatography using a 30 cm $C_{18}$ reverse-phase column and a UV detector set at 280 nm. The mobile phase in volume percent is 2.5 percent acetic acid, 50 percent acetonitrile, and 47.5 percent water which is passed through the column at a flow rate of 2 mL/minute.

(ii) *Preparations*—(A) *Preparation of test chemical solution.* Prepare homogeneous solutions with the chemical at less than one-half of its solubility in water and at a concentration such that the absorbance is less than 0.05 in the photolysis reaction vessel at wavelengths greater than 290 nm. For very hydrophobic chemicals, it is difficult and time consuming to prepare aqueous solutions. To facilitate the preparation of aqueous solutions containing very hydrophobic chemicals and to allow for easier analytical measurement procedures, the following procedure may be used to aid in the dissolution of the chemical. Dissolve the pure chemical in reagent grade acetonitrile. Add pure water as described under Test Conditions, in paragraph (b)(2)(i)(B) of this section, or buffer solution as described under Preparations, in paragraph (b)(2)(ii)(B) of this section, for chemical substances which ionize or protonate, to an aliquot of the acetonitrile solution. Do not exceed one volume-percent of acetonitrile in the final solution. Place the reaction solution in the appropriate photolysis reaction tubes as described in paragraph (b)(2)(i)(A) of this section.

(B) *Preparation of buffer solutions.* Prepare buffer solutions according to the procedures outlined in paragraph (b)(1)(i) of this section using reagent grade chemicals and pure water as described under Test Conditions, in paragraph (b)(2)(i)(B) of this section.

(C) *Preparation of actinometer solution.* (*1*) Using the test chemical photolysis rate constant, $k_p^c$, determined in Tier 2, Phase 1, and the variable $k_a^c$ listed in Table 2 under paragraph (c)(3) of this section, the molar concentration of pyridine needed to adjust the rate of disappearance of p-nitroacetophenone (PNAP) to match the rate of disappearance of the test chemical can be obtained from equation 11 under paragraph (b)(2)(i)(H)(*2*) of this section. The variable $k_a^*$ ($=\Sigma\epsilon_\lambda^* L_\lambda$) is equal to the day-average rate constant for sunlight absorption by PNAP which changes with season and latitude. The value of $k_a^*$ is selected from Table 2 under paragraph (c)(3) of this section for the season nearest the mid-experiment date of the Tier 2, Phase 1, studies and the decadic latitude nearest the latitude of the experimental site.

(*2*) Once the molar concentration of pyridine [PYR] has been determined, an actinometer solution can be prepared as follows. Dissolve 0.165 gm. of PNAP in 100 mL of acetonitrile (0.01 M). Add 1 mL of this solution to a 1 liter volumetric flask. Add to the volumetric flask the mass in grams, or the volume (V) of pyridine at 20° C, obtained from the equations

*Equation 14*

$$\text{mass(grams)} = 79.1\ [\text{PYR}]$$

$$V(\text{mL}) = 80.6\ [\text{PYR}].$$

Fill the volumetric flask with pure water in paragraph (b)(2)(i)(B) of this section to give 1 liter of solution and shake vigorously to make sure that the solution is homogeneous. The PNAP/PYR solution should be wrapped with aluminum foil and kept from bright light.

(iii) *Performance of the tests*—(A) *Phase 1 experiments.* (*1*) For all experiments, prepare an aqueous solution of the chemical substance, as described in paragraph (b)(2)(ii)(A) of this section, and a sufficient number of samples in quartz or borosilicate glass tubes to perform all the required tests. Fill the tubes as completely as possible and seal them. Prepare two control samples in the absence of ultraviolet light and totally exclude light by wrapping the tubes with aluminum foil or by other suitable methods. These samples are analyzed for the chemical substance immediately after completion of the experiment to measure the loss of chemical in the absence of light. Place the samples, including the controls, outdoors in an area free of shade and reflections of sunlight from windows and buildings. Place the samples on a black, nonreflective background and inclined at approximately 30° from the horizontal with upper end pointing due north (in the northern hemisphere). Conduct the photolysis experiments during a frost-free time of year (e.g., May, June, July, August, or September in the northern hemisphere—weather permitting) and start the experiments initially at noon (1,200 hours). Record the date and time the experiment was begun, the date and time completed, the time of sunrise and sunset on all days when photolysis experiments were performed, the times exposure was stopped and restarted for intermittent exposure, the weather conditions during the period, and the latitude of the site. For chemical substances that ionize or protonate, carry out photolysis experiments at the required pHs as described under Test Conditions under paragraph (b)(2)(i)(D) of this section.

(*2*) If a significant loss of test chemical has occurred in the control samples, determine the cause and eliminate or minimize the loss. If hydrolysis is found to be significant, hydrolysis studies should be carried out first under paragraph (b)(2)(i)(F) of this section.

(*3*) Use one of the following procedures, depending on how fast the chemical substance photolyzes.

(*i*) *Procedure 1.* If the chemical substance transforms 50 to 80 percent within 28 days, measure the concentration of the chemical substance, in duplicate, at time t=0 and periodically (at least four data points at approximately equal time intervals) at noon (1200 hours) until at least 50 percent of the substance has been consumed. As a simplification, the sampling times can be estimated as the photolysis experiments progress. Determine the concentration of test chemical from two, freshly opened, reaction tubes for each time point. Determine the concentration in each of the two control solutions as soon as the photolysis experiments are completed.

(*ii*) *Procedure 2.* If the chemical substance transforms in the range of 20 to 50 percent in 28 days, determine the concentration of the chemical substance, in duplicate, at time t=0. Determine the concentration of the chemical in the two separate reaction tubes and the two control tubes after 28 days of photolysis.

(*iii*) *Procedure 3.* For chemical substances that transform in sunlight 50 to 80 percent within one or two days, place the samples outside at noon (1200 hours) and analyze two samples for the concentration of the chemical substance at $t=0$, and in two, freshly opened, reaction tubes at noon (1200 hours) the next day, and again, in two, freshly opened, reaction tubes at noon (1200 hours) the second day. Determine the concentration of the test chemical in each of the two control solutions after the first day of photolysis and as soon as the photolysis experiments have been completed on the second day.

(*iv*) *Analytical methodology.* Select an analytical method which is most applicable to the analysis of the specific chemical being tested under paragraph (b)(2)(i)(K) of this section.

(B) *Phase 2 experiments.* (*1*) Using the test chemical photolysis rate constant, $k^c_p$, determined in Tier 2, Phase 1, prepare an actinometer solution, as described in paragraph (b)(2)(ii)(C) of this section and a sufficient number of samples in quartz tubes to perform all the required tests. Fill all the tubes as completely as possible, seal them, and cover them with aluminum foil as soon as possible after preparation. Prepare an aqueous solution of test chemical, as described in paragraph (b)(2)(ii)(A) of this section, and a sufficient number of samples in quartz or borosilicate tubes to perform all the required tests. Fill these tubes as completely as possible, seal them, and cover them with aluminum foil as soon as possible after preparation. Place all the samples outdoors in an area free of shade and reflections of sunlight from windows and buildings. Place the samples on a black, nonreflective background and inclined at approximately 30° from the horizontal with the upper end pointing due north (in the northern hemisphere). Remove the foil from all samples except for the test chemical control solutions and the actinometer control solutions at noon (1200 hours). Based on the results of the Phase 1 experiments, determine the concentration of test chemical and actinometer (PNAP), in triplicate, at time $t=0$ and periodically (at least five data points at approximately equal time intervals). Determine the concentration of PNAP in the three actinometer control solutions and the concentration of test chemical in the three control solutions for each time point.

(*2*) Select an analytical method which is most applicable to the analysis of the specific chemical tested, in paragraph (b)(2)(i)(K) of this section and follow the procedure given in paragraph (b)(2)(i)(K) of this section for the analysis of PNAP.

(c) *Data and reporting*—(1) *Tier 1 Test: UV-Visible Absorption Spectra—Estimation of Aqueous Photolysis Maximum Rate Constant and Minimum Half-Life in Sunlight*—(i) *Treatment of results.* (A) The molar absorptivity can be determined from the absorption spectra using the expression.

*Equation 15*

$$\epsilon_\lambda^c = A_\lambda / Cl,$$

where $A_\lambda$ is the absorbance at wavelength $_\lambda$, C is the molar concentration of test chemical, and l is the cell pathlength in centimeters. The molar absorptivity of the chemical should be determined for the wavelengths listed in Table 1 under paragraph (c)(3) of this section for a solution of concentration C and in a cell with pathlength. *l.* If the absorption curve is flat within the interval around the wavelength $_\lambda$center, $\epsilon_\lambda$ may be determined from the absorbance $A_\lambda$ at $_\lambda$center using equation 15. If a large change in absorbance occurs within this interval, obtain an average absorbance $A_\lambda$ at $_\lambda$center based on the absorbances at the two boundaries of the interval. Calculate an average $\epsilon_\lambda$ using the average value of $A_\lambda$ in equation 15. Determine the molar absorptivity for each replicate and calculate a mean value.

(B) Using the molar absorptivities obtained from the spectra and the values of the $L_\lambda$ from Tables 3 to 6 under paragraph (c)(3) of this section, the maximum rate constant $(k_{pE})_{max}$ can be calculated at a specific latitude and season of the year using equation 7 under paragraph (a)(3)(v) of this section. The minimum half-life, $(t_{1/2E})_{min}$.

can then be calculated using this $(k_{pE})_{max}$. in equation 8 under paragraph (a)(3)(v) of this section.

(C) Two hypothetical examples are presented in paragraph (c)(4)(i) of this section to illustrate how the test data obtained in the first-tier screening test can be used.

(ii) *Test data report.* (A) Submit the original chart, or photocopy, containing a plot of absorbance of test chemical vs. wavelength plus the baseline. Spectra should include a readable wavelength scale, preferably marked at 10 nm intervals. Each spectrum should be clearly marked with the test conditions.

(B) Report the concentration of the test chemical solution, the type of absorption cell used (quartz or borosilicate glass) and the pathlength.

(C) Report $A_\lambda$ and $\epsilon_\lambda$ at $_\lambda$center for each replicate and the mean value.

(D) Report $(k_{pE})_{max}$. and $(t_{1/2E})_{min}$. for the summer and winter solstices using the appropriate $L_\lambda$ values from Tables 3–6 closest to the latitude of the chemical manufacturing site.

(E) Report the identity and compositon of the solvent used in the spectral absorption study.

(F) For ionizable chemicals, report its $pK_a$. Report the type and concentration of the buffers employed for each pH. Report the pHs in which the photolysis experiments were carried out.

(G) Describe the method employed in determining the test chemical's concentration.

(H) Report the name, structure, and purity of the test chemical.

(I) Submit a recent test spectrum on appropriate reference chemicals for photometric and wavelength accuracy.

(J) Report the name and model of the spectrophotometer used.

(K) Report the various control settings employed with the spectrophotometer. These might include scan speed, slit width, gain, etc.

(2) *Tier 2 Test: Aqueous photolysis in sunlight*—(i) *Phase 1 experiments*—(A) *Treatment of results.* (*1*) If a small loss of test substance in the control tubes has occurred, use this data to make corrections to the measured photolysis rate in paragraph (b)(2)(i)(F) of this section. Note the site of photolysis and its latitude and the weather conditions. For Procedures 1 and 2 note the dates and times of actual exposure including times of sunrise and sunset and, in case the cells are moved to prevent freezing or for other reasons, make sure that these times are recorded and that the cells are kept in a dark place when exposure is not in progress.

(*i*) For chemical substances which transform 50 to 80 percent within 28 days, use a concentration $C_t$, which corresponds to less than 50 percent of the initial concentration of chemical substance remaining, and the corresponding time t, in days, along with the initial molar concentration $C_o$, in Equation 5 to calculate $k_p$ in days$^{-1}$. From the analysis of the two samples at time t=0 and t, calculate a mean value of $C_o$ and $C_t$, respectively, and a value of $k_p$. If a slight loss of chemical has been detected in the controls, then calculate a rate constant as follows: Calculate an average concentration $C_t$, based on the duplicate measurements of concentration in the controls. Use this concentration along with the average initial concentration in Equation 5 and calculate a rate constant $k_{loss}$. Using this rate constant along with the observed rate constant, the corrected rate constant is then

*Equation 16*

$$k_p = (k_p)_{obs} - k_{loss}.$$

Calculate the half-life, $t_{1/2}$ using the corrected $k_p$ value in Equation 6 under paragraph (a)(3)(iv) of this section.

(*ii*) For chemical substances which transform 20 to 50 percent in 28 days, use the mean concentration $C_t$ remaining at t=28 days along with the mean value of $C_o$ to calculate $k_p$. Use the same procedure as described above to calculate the value of $k_p$ and $t_{1/2}$. If less than 20 percent of the chemical substance degrades in 28 days, report the mean concentration of $C_t$ and $C_o$. In this case the apparent half-life is reported as greater than 3 months.

(*iii*) For chemical substances which transform 50 percent or more in the first day, as described in Procedure 3, calculate a full day $k_p$ value using the mean concentration $C_t$ of chemical substance remaining at noon (1200 hours) after the first day along with the mean value of $C_o$ using Equation 5 under paragraph (a)(3)(iv) of this section. For chemical substances which degrade less than 50 percent at noon (1200 hours) after the first day but 50 percent or more at noon (1200 hours) the second day, calculate $k_p$ using the mean concentration of chemical substances remaining at noon (1200 hours) the second day. Calculate the half-life, $t_{1/2}$, using the mean value of $k_p$ in Equation 6 under paragraph (a)(3)(iv) of this section. If a small loss of test substance in the control tubes has occurred, use this data to make corrections to the measured photolysis rate as described. Note the dates of photolysis, the latitude, and the site.

(*2*) A hypothetical example is presented in paragraph (c)(4)(ii) of this section, to illustrate how the test data obtained in the Tier 2, Phase 1, test method can be used.

(B) *Specific analytical and recovery procedures.* (*1*) Provide a detailed description or reference for the analytical procedures used, including the calibration data and precision.

(*2*) If extraction methods were used to separate the solute from the aqueous solution, provide a description of the extraction method as well as the recovery data.

(C) *Other test conditions.* (*1*) Report the size, approximate cell wall thickness, and type of glass used for the reaction tubes.

(*2*) Report the initial pH of all test solutions, if appropriate.

(*3*) For all procedures, report the dates of photolysis, the time of sunrise and sunset on each photolysis day, the site of photolysis and its latitude, and the weather conditions. For Procedures 1 and 2 submit the dates and times of actual exposure, and the duration of exposure, and, for intermittent exposure, the fraction of each day during which photolysis occurred.

(*4*) If acetonitrile was used to solubilize the test substance, report the percent, by volume.

(*5*) If a significant loss of test chemical occurred in the control solution, indicate the causes and how they were eliminated or minimized.

(D) *Test data report.* (*1*) For each photolysis experiment, report:

(*i*) The initial molar concentration of test chemical ($C_o$) of each replicate and the mean value.

(*ii*) The molar concentration of test chemical for each replicate and the mean value for each time point t.

(*iii*) The molar concentration of each replicate control sample and the mean value after completion of the photolysis experiments.

(*2*) For Procedure 1, 2, or 3, report the value of $k_p$. If small losses of chemical are observed, report $(k_p)_{obs.}$, $k_{loss}$ and $k_p$. Report the half-life ($t_{1/2}$) calculated using the value of $k_p$.

(ii) *Phase 2 experiments*—(A) *Treatment of results.* (*1*) The objectives of this set of experiments is to determine the sunlight reaction quantum yield, $\phi^c_E$, for a specific test chemical. $\phi^c_E$ can be calculated using Equation 10 under paragraph (a)(3)(vii) of this section,

$$\phi^c_E = \frac{k^c_p}{k^a_p} \, \frac{\Sigma \epsilon^a_\lambda L_\lambda}{\Sigma \epsilon^c_\lambda L_\lambda} \, \phi^a_E$$

by the following steps:

(*i*) Determine the ratio of the rate constants, $k^a_p/k^c_p$, as described in paragraph (b)(2)(i)(H) of this section using Equation 12. If a slight loss of test chemical or actinometer (PNAP) was detected in the controls at any time t, then employ the following procedure. Consider, as an example, the loss of test chemical in the control at time t. Using the average concentration of the test chemical in the controls from the replicates at time t and the average initial concentration, calculate $\ln(C_o/C_t)^c_{loss}$. Using the average concentration of test chemical from the replicates after photolysis time t, calculate $\ln(C_o/C_t)^c_{obs.}$. The corrected term is then

*Equation 17*

$$\ln(C_o/C_t)^c_{corr.} = \ln(C_o/C_t)^c_{loss}$$

The same procedure can be applied to obtain a corrected term from the actinometer (PNAP). Using the corrected terms for test chemical and/or actinometer in Equation 12 under paragraph (b)(2)(i)(H)(*3*) of this section, determine the ratio of the rate constants ($k^a_p/k^c_p$) as described in paragraph (b)(2)(i)(H) of this section.

(*ii*) Determine the quantum yield of the actinometer, $\phi^a_E$, using Equation 9 and the molar concentration of pyridine [PYR] present in the actinometer.

(*iii*) Determine the value of $\Sigma\epsilon^c_\lambda L_\lambda$ for the test chemical as follows: the molar absorptivities, $\epsilon^c_\lambda$, have been determined by the procedure given in paragraph (b)(1) of this section and the results have been tabulated according to paragraph (c)(1)(ii) of this section. Choose the appropriate $L_\lambda$ values (Tables 3 to 6 under paragraph (c)(3) of this section) that correspond to the season closest to the season in which the Phase 2 experiments were performed and to the latitude nearest the latitude of the experimental site. Calculate the product of $\epsilon^c_\lambda$ and $L_\lambda$ for each wavelength interval where $\epsilon_\lambda$ has a nonzero value. Sum the products of $\epsilon^a_\lambda L_\lambda$ over all wavelength intervals.

(*iv*) Determine the value of $\Sigma\epsilon_\lambda{}^a L_\lambda$, for the actinometer, as follows: These values have been calculated and are given in Table 2 under paragraph (c)(3) of this section. Choose the appropriate value that corresponds to the season closest to the season in which the Phase 2 experiments were performed and to the latitude nearest the latitude of the experimental site.

(*v*) Substitute the values of $k^a_p/k^c_p$, $\phi^a_E$, $\Sigma\epsilon^a_\lambda L_\lambda$, and $\Sigma\epsilon^c_\lambda L_\lambda$ in Equation 10 under paragraph (a)(3)(iii) of this section and calculate $\phi^c_E$, the quantum yield of the test chemical in the environment (i.e., in sunlight).

(*2*) Once $\phi^c_E$ has been determined, equation 4 under paragraph (a)(3)(iii) of this section can be used to calculate $k_{pE}$ at any season of the year and latitude using the measured values of the molar absorptivities, $\epsilon^c_\lambda$, and the appropriate $L_\lambda$ values (Tables 3 to 6 under paragraph (c)(3) of this section). The half-life can then be calculated using $k_{pE}$ in Equation 2 under paragraph (a)(3)(ii) of this section.

(*3*) A hypothetical example is presented in paragraph (c)(4)(iii) of this section, to illustrate how the test data obtained in the Tier 2, Phase 1, test method can be used.

(B) *Other test conditions.* (*1*) Report the size, approximate cell wall thickness, and type of glass used for tubes to hold the test chemical and actinometer solutions.

(*2*) Report the initial pH of all test chemical solutions, if appropriate, and the type and concentration of the buffers employed for each pH.

(*3*) If acetonitrile was used to solubilize the test chemical, report the percent, by volume, of the acetonitrile, which was used.

(*4*) If significant loss of test chemical occurred in the control solution, indicate the causes and how they were eliminated or minimized.

(C) *Test data report.* (*1*) Report the initial molar concentration of chemical ($C_0$) of each replicate and the mean value.

(*2*) Report the initial molar concentration of PNAP and the molar concentration of pyridine used in the actinometer.

(*3*) Report the time and date the sunlight photolysis experiments were started, the time and date the experiments were completed, and the elapsed photolysis time in days.

(*4*) For each time point, report the three separate values for the molar concentration of test chemical and PNAP and the mean values.

(*5*) For each time point, report the three separate values of the molar concentration of test chemical and PNAP for the controls and the mean values.

(*6*) Tabulate and report the following data: t, $\ln(C_0/C_t)^c$, and $\ln(C_0/C_t)^a$. From the linear regression analysis, report the ratio of the rate constants, $k_p{}^c/k_p{}^a$, and the correlation coefficient.

(*7*) If loss of test chemical and/or actinometer was observed during photolysis, then report the data $\ln(C_0/C_t)_{corr.}$, $\ln(C_0/C_t)_{obs.}$, $\ln(C_0/C_t)_{loss}$ for the test chemical and/or actinometer at each time t. From the linear regression analysis of $\ln(C_0/C_t)^c{}_{corr.}$ and $\ln(C_0/C_t)^a{}_{corr.}$, report the ratio of the rate constants, $k_p{}^c/k_p{}^a$ and the correlation coefficient.

(*8*) Report the reaction quantum yield of the actinometer ($\phi^a_E$).

(*9*) Report the value of $k_s{}^a$ for the actinometer corresponding to the season closest to the season in which the photolysis experiments were carried out and to the latitude nearest the latitude of the experimental site.

(*10*) Tabulate the values of λcenter, $\epsilon_\lambda^c$, $L_\lambda$, and $\epsilon_\lambda^c L_\lambda$ for the test chemical corresponding to the season closest to the season in which the photolysis experiments were carried out and to the latitude nearest the latitude of the experimental site.

(*11*) Report the value $-\Sigma \epsilon_\lambda^c L_\lambda$ for the test chemical from step 10.

(*12*) Report the reaction quantum yield of the test chemical.

(*13*) Report $k_{pE}$ and $t_{1/2E}$ for the summer and winter seasons using the appropriate $L_\lambda$ values from Tables 3-6 under paragraph (c)(3) of this section closest to the latitude of the chemical manufacturing site.

(*14*) For chemicals that ionize, report the data for steps 1-13 for the experiments at the required pHs.

(3) *Tables of solar irradiance and related tables.*

TABLE 1—WAVELENGTH CENTER AND INTERVALS FOR $L_\lambda$

| λ center (nm) | Interval from (nm) | Range to (nm) | Δλ (nm) |
|---|---|---|---|
| 297.5 | 296.2 | 298.7 | 2.5 |
| 300.0 | 298.7 | 301.2 | 2.5 |
| 302.5 | 301.2 | 303.7 | 2.5 |
| 305.0 | 303.7 | 306.2 | 2.5 |
| 307.5 | 306.2 | 308.7 | 2.5 |
| 310.0 | 308.7 | 311.2 | 2.5 |
| 312.5 | 311.2 | 313.7 | 2.5 |
| 315.0 | 313.7 | 316.2 | 2.5 |
| 317.5 | 316.2 | 318.7 | 2.5 |
| 320.0 | 318.7 | 321.2 | 2.5 |
| 323.1 | 321.2 | 325.0 | 3.8 |
| 330.0 | 325.0 | 335.0 | 10.0 |
| 340.0 | 335.0 | 345.0 | 10.0 |
| 350 | 345.0 | 355.0 | 10.0 |
| 360 | 355.0 | 365.0 | 10.0 |
| 370 | 365.0 | 375.0 | 10.0 |
| 380 | 375.0 | 385.0 | 10.0 |
| 390 | 385.0 | 395.0 | 10.0 |
| 400 | 395.0 | 405.0 | 10.0 |
| 410 | 405.0 | 415.0 | 10.0 |
| 420 | 415.0 | 425.0 | 10.0 |
| 430 | 425.0 | 435.0 | 10.0 |
| 440 | 435.0 | 445.0 | 10.0 |
| 450 | 445.0 | 455.0 | 10.0 |
| 460 | 455.0 | 465.0 | 10.0 |
| 470 | 465.0 | 475.0 | 10.0 |
| 480 | 475.0 | 485.0 | 10.0 |
| 490 | 485.0 | 495.0 | 10.0 |
| 500 | 495.0 | 505.0 | 10.0 |
| 525 | 512.5 | 537.5 | 25 |
| 550 | 537.5 | 562.5 | 25 |
| 575 | 562.5 | 587.5 | 25 |
| 600 | 587.5 | 612.5 | 25 |
| 625 | 612.5 | 637.5 | 25 |
| 650 | 637.5 | 662.5 | 25 |
| 675 | 662.5 | 687.5 | 25 |

TABLE 1—WAVELENGTH CENTER AND INTERVALS FOR $L_\lambda$—Continued

| λ center (nm) | Interval from (nm) | Range to (nm) | Δλ (nm) |
|---|---|---|---|
| 700 | 687.5 | 712.5 | 25 |
| 750 | 725.0 | 775.0 | 50 |
| 800 | 775.0 | 825.0 | 50 |

TABLE 2—DAY AVERAGED RATE CONSTANT ($k^a_a$)[1] FOR SUNLIGHT ABSORPTION BY PNAP AS A FUNCTION OF SEASON AND DECADIC LATITUDE

| Latitude (degrees north) | Season | | | |
|---|---|---|---|---|
| | Spring | Summer | Fall | Winter |
| 20 | 515 | 551 | 409 | 327 |
| 30 | 483 | 551 | 333 | 233 |
| 40 | 431 | 532 | 245 | 139 |
| 50 | 382 | 496 | 154 | 64 |

[1] $k^a_a = \Sigma \epsilon^a_\lambda L_\lambda$ in day$^{-1}$.

TABLE 3—$L_\lambda$ VALUES FOR LATITUDE 20° N.[1] [2] [3]

| λ center (nm) | Spring | Summer | Fall | Winter |
|---|---|---|---|---|
| 297.5 | 1.10(−4) | 1.52(−4) | 7.77(−5) | 3.71(−5) |
| 300.0 | 4.06(−4) | 5.26(−4) | 2.96(−4) | 1.62(−4) |
| 302.5 | 1.10(−3) | 1.35(−3) | 8.21(−4) | 4.99(−4) |
| 305.0 | 2.37(−3) | 2.79(−3) | 1.79(−3) | 1.17(−3) |
| 307.5 | 4.24(−3) | 4.86(−3) | 3.24(−3) | 2.25(−3) |
| 310.0 | 6.65(−3) | 7.45(−3) | 5.13(−3) | 3.72(−3) |
| 312.5 | 9.42(−3) | 1.04(−2) | 7.33(−3) | 5.47(−3) |
| 315.0 | 1.24(−2) | 1.35(−2) | 9.66(−3) | 7.40(−3) |
| 317.5 | 1.54(−2) | 1.66(−2) | 1.21(−2) | 9.36(−3) |
| 320.0 | 1.82(−2) | 1.96(−2) | 1.44(−2) | 1.13(−2) |
| 323.1 | 3.23(−2) | 3.45(−2) | 2.55(−2) | 2.04(−2) |
| 330.0 | 1.10(−1) | 1.17(−1) | 8.75(−2) | 7.06(−2) |
| 340.0 | 1.37(−1) | 1.45(−1) | 1.10(−1) | 9.02(−2) |
| 350.0 | 1.52(−1) | 1.60(−1) | 1.22(−1) | 1.01(−1) |
| 360.0 | 1.67(−1) | 1.76(−1) | 1.35(−1) | 1.12(−1) |
| 370.0 | 1.78(−1) | 1.88(−1) | 1.45(−1) | 1.21(−1) |
| 380.0 | 1.89(−1) | 2.00(−1) | 1.55(−1) | 1.30(−1) |
| 390.0 | 1.79(−1) | 1.89(−1) | 1.46(−1) | 1.22(−1) |
| 400.0 | 2.57(−1) | 2.71(−1) | 2.09(−1) | 1.75(−1) |
| 410.0 | 3.38(−1) | 3.57(−1) | 2.76(−1) | 2.31(−1) |
| 420.0 | 3.47(−1) | 3.67(−1) | 2.84(−1) | 2.38(−1) |
| 430.0 | 3.35(−1) | 3.54(−1) | 2.74(−1) | 2.30(−1) |
| 440.0 | 3.96(−1) | 4.19(−1) | 3.25(−1) | 2.72(−1) |
| 450.0 | 4.45(−1) | 4.70(−1) | 3.65(−1) | 3.07(−1) |
| 460.0 | 4.50(−1) | 4.75(−1) | 3.70(−1) | 3.11(−1) |
| 470.0 | 4.65(−1) | 4.91(−1) | 3.83(−1) | 3.22(−1) |
| 480.0 | 4.76(−1) | 5.03(−1) | 3.92(−1) | 3.31(−1) |
| 490.0 | 4.50(−1) | 4.76(−1) | 3.72(−1) | 3.13(−1) |
| 500.0 | 4.59(−1) | 4.85(−1) | 3.80(−1) | 3.20(−1) |
| 525.0 | 1.21 | 1.28 | 1.00 | 8.48(−1) |
| 550.0 | 1.26 | 1.33 | 1.05 | 8.83(−1) |
| 575.0 | 1.27 | 1.35 | 1.06 | 8.92(−1) |
| 600.0 | 1.29 | 1.36 | 1.07 | 9.05(−1) |
| 625.0 | 1.29 | 1.37 | 1.08 | 9.15(−1) |
| 650.0 | 1.30 | 1.38 | 1.09 | 9.24(−1) |
| 675.0 | 1.30 | 1.38 | 1.09 | 9.27(−1) |
| 700.0 | 1.29 | 1.36 | 1.08 | 9.21(−1) |
| 750.0 | 2.48 | 2.62 | 2.08 | 1.78 |

### TABLE 3—$L_\lambda$ VALUES FOR LATITUDE 20° N.[1][2][3]—Continued

| λ center (nm) | Spring | Summer | Fall | Winter |
|---|---|---|---|---|
| 800.0 | 2.38 | 2.51 | 2.00 | 1.71 |

[1] Units of $L_\lambda$ are $10^{-3}$ einsteins $cm^{-2}$ $day^{-1}$. Multiplication of $L_\lambda$ by $\epsilon_\lambda$ in units of $molar^{-1}$ $cm^{-1}$ gives rate constants in units of $day^{-1}$.

[2] The second number in the columns in parenthesis is the power of ten by which the first number is multiplied.

[3] Based on the GC SOLAR program.

### TABLE 4—$L_\lambda$ VALUES FOR LATITUDE 30° N.[1][2][3]

| λcenter (nm) | Spring | Summer | Fall | Winter |
|---|---|---|---|---|
| 297.5 | 5.73(−5) | 1.09(−4) | 3.18(−5) | 6.78(−6) |
| 300.0 | 2.50(−4) | 4.11(−4) | 1.46(−4) | 4.23(−5) |
| 302.5 | 7.65(−4) | 1.14(−3) | 4.64(−4) | 1.71(−4) |
| 305.0 | 1.79(−3) | 2.46(−3) | 1.12(−3) | 4.95(−4) |
| 307.5 | 3.43(−3) | 4.45(−3) | 2.19(−3) | 1.11(−3) |
| 310.0 | 5.64(−3) | 7.02(−3) | 3.67(−3) | 2.04(−3) |
| 312.5 | 8.27(−3) | 1.00(−2) | 5.46(−3) | 3.26(−3) |
| 315.0 | 1.12(−2) | 1.32(−2) | 7.43(−3) | 4.69(−3) |
| 317.5 | 1.41(−2) | 1.64(−2) | 9.48(−3) | 6.21(−3) |
| 320.0 | 1.70(−2) | 1.95(−2) | 1.15(−2) | 7.76(−3) |
| 323.1 | 3.04(−2) | 3.46(−2) | 2.07(−2) | 1.43(−2) |
| 330.0 | 1.05(−1) | 1.18(−1) | 7.23(−2) | 5.17(−2) |
| 340.0 | 1.33(−1) | 1.48(−1) | 9.23(−2) | 6.75(−2) |
| 350.0 | 1.47(−1) | 1.63(−1) | 1.03(−1) | 7.65(−2) |
| 360.0 | 1.62(−1) | 1.80(−1) | 1.15(−1) | 8.60(−2) |
| 370.0 | 1.73(−1) | 1.91(−1) | 1.24(−1) | 9.31(−2) |
| 380.0 | 1.84(−1) | 2.04(−1) | 1.33(−1) | 1.01(−1) |
| 390.0 | 1.74(−1) | 1.93(−1) | 1.25(−1) | 9.39(−2) |
| 400.0 | 2.50(−1) | 2.77(−1) | 1.79(−1) | 1.35(−1) |
| 410.0 | 3.29(−1) | 3.64(−1) | 2.36(−1) | 1.79(−1) |
| 420.0 | 3.38(−1) | 3.74(−1) | 2.43(−1) | 1.84(−1) |
| 430.0 | 3.26(−1) | 3.61(−1) | 2.35(−1) | 1.78(−1) |
| 440.0 | 3.86(−1) | 4.26(−1) | 2.79(−1) | 2.12(−1) |
| 450.0 | 4.34(−1) | 4.79(−1) | 3.14(−1) | 2.39(−1) |
| 460.0 | 4.39(−1) | 4.85(−1) | 3.19(−1) | 2.42(−1) |
| 470.0 | 4.54(−1) | 5.01(−1) | 3.30(−1) | 2.51(−1) |
| 480.0 | 4.65(−1) | 5.13(−1) | 3.38(−1) | 2.58(−1) |
| 490.0 | 4.40(−1) | 4.85(−1) | 3.20(−1) | 2.44(−1) |
| 500.0 | 4.49(−1) | 4.95(−1) | 3.27(−1) | 2.50(−1) |
| 525.0 | 1.18 | 1.31 | 8.67(−1) | 6.61(−1) |
| 550.0 | 1.23 | 1.36 | 9.03(−1) | 6.87(−1) |
| 575.0 | 1.24 | 1.37 | 9.11(−1) | 6.93(−1) |
| 600.0 | 1.25 | 1.38 | 9.24(−1) | 7.04(−1) |
| 625.0 | 1.26 | 1.39 | 9.34(−1) | 7.15(−1) |
| 650.0 | 1.27 | 1.40 | 9.45(−1) | 7.27(−1) |
| 675.0 | 1.28 | 1.40 | 9.48(−1) | 7.32(−1) |
| 700.0 | 1.27 | 1.39 | 9.42(−1) | 7.31(−1) |
| 750.0 | 2.44 | 2.67 | 1.82 | 1.41 |
| 800.0 | 2.34 | 2.57 | 1.75 | 1.37 |

[1] Units of $L_\lambda$ are $10^{-3}$ einsteins $cm^{-2}$ $day^{-1}$. Multiplication of $L_\lambda$ by $\epsilon_\lambda$ in units of $molar^{-1}$ $cm^{-1}$ gives rate constants in units of $day^{-1}$.

[2] The second number in the columns in parenthesis is the power of ten by which the first number is multiplied.

[3] Based on the GC SOLAR program.

### TABLE 5—$L_\lambda$ VALUES FOR LATITUDE 40° N.[1][2][3]

| λcenter (nm) | Spring | Summer | Fall | Winter |
|---|---|---|---|---|
| 297.5 | 1.85(−5) | 6.17(−5) | 7.83(−6) | 5.49(−7) |
| 300.0 | 1.06(−4) | 2.70(−4) | 4.76(−5) | 5.13(−6) |

### TABLE 5—$L_\lambda$ VALUES FOR LATITUDE 40° N.[1][2][3]—Continued

| λcenter (nm) | Spring | Summer | Fall | Winter |
|---|---|---|---|---|
| 302.5 | 3.99(−4) | 8.30(−4) | 1.89(−4) | 3.02(−5) |
| 305.0 | 1.09(−3) | 1.95(−3) | 5.40(−4) | 1.19(−4) |
| 307.5 | 2.34(−3) | 3.74(−3) | 1.19(−3) | 3.36(−4) |
| 310.0 | 4.17(−3) | 6.17(−3) | 2.19(−3) | 7.53(−4) |
| 312.5 | 6.51(−3) | 9.07(−3) | 3.47(−3) | 1.39(−3) |
| 315.0 | 9.18(−2) | 1.22(−2) | 4.97(−3) | 2.22(−3) |
| 317.5 | 1.20(−2) | 1.55(−2) | 6.57(−3) | 3.19(−3) |
| 320.0 | 1.48(−2) | 1.87(−2) | 8.16(−3) | 4.23(−3) |
| 323.1 | 2.71(−2) | 3.35(−2) | 1.51(−2) | 8.25(−3) |
| 330.0 | 9.59(−2) | 1.16(−1) | 5.44(−2) | 3.16(−2) |
| 340.0 | 1.23(−1) | 1.46(−1) | 7.09(−2) | 4.31(−2) |
| 350.0 | 1.37(−1) | 1.62(−1) | 8.04(−2) | 4.98(−2) |
| 360.0 | 1.52(−1) | 1.79(−1) | 9.02(−2) | 5.68(−2) |
| 370.0 | 1.63(−1) | 1.91(−1) | 9.77(−2) | 6.22(−2) |
| 380.0 | 1.74(−1) | 2.04(−1) | 1.06(−1) | 6.78(−2) |
| 390.0 | 1.64(−1) | 1.93(−1) | 9.86(−2) | 6.33(−2) |
| 400.0 | 2.36(−1) | 2.76(−1) | 1.42(−1) | 9.11(−2) |
| 410.0 | 3.10(−1) | 3.64(−1) | 1.87(−1) | 1.20(−1) |
| 420.0 | 3.19(−1) | 3.74(−1) | 1.93(−1) | 1.24(−1) |
| 430.0 | 3.06(−1) | 3.61(−1) | 1.87(−1) | 1.20(−1) |
| 440.0 | 3.65(−1) | 4.26(−1) | 2.22(−1) | 1.43(−1) |
| 450.0 | 4.11(−1) | 4.80(−1) | 2.51(−1) | 1.61(−1) |
| 460.0 | 4.16(−1) | 4.85(−1) | 2.54(−1) | 1.64(−1) |
| 470.0 | 4.30(−1) | 5.02(−1) | 2.63(−1) | 1.69(−1) |
| 480.0 | 4.40(−1) | 5.14(−1) | 2.70(−1) | 1.74(−1) |
| 490.0 | 4.16(−1) | 4.86(−1) | 2.56(−1) | 1.65(−1) |
| 500.0 | 4.25(−1) | 4.96(−1) | 2.62(−1) | 1.68(−1) |
| 525.0 | 1.12 | 1.31 | 6.93(−1) | 4.45(−1) |
| 550.0 | 1.16 | 1.36 | 7.21(−1) | 4.61(−1) |
| 575.0 | 1.17 | 1.37 | 7.22(−1) | 4.61(−1) |
| 600.0 | 1.18 | 1.38 | 7.39(−1) | 4.69(−1) |
| 625.0 | 1.20 | 1.40 | 7.50(−1) | 4.82(−1) |
| 650.0 | 1.21 | 1.41 | 7.62(−1) | 4.95(−1) |
| 675.0 | 1.22 | 1.41 | 7.68(−1) | 5.03(−1) |
| 700.0 | 1.21 | 1.40 | 7.66(−1) | 5.05(−1) |
| 750.0 | 2.33 | 2.69 | 1.48 | 9.84(−1) |
| 800.0 | 2.25 | 2.59 | 1.43 | 9.56(−1) |

[1] Units of $L_\lambda$ are $10^{-3}$ einsteins $cm^{-2}$ $day^{-1}$. Multiplication of $L_\lambda$ $\epsilon_\lambda$ in the units of $molar^{-1}$ $cm^{-1}$ gives the rate constant in units of $day^{-1}$.

[2] The second number in the columns in parenthesis is the power of ten by which the first number is multiplied.

[3] Based on the GC SOLAR program.

### TABLE 6—$L_\lambda$ VALUES FOR LATITUDE 50° N.[1][2][3]

| λcenter (nm) | Spring | Summer | Fall | Winter |
|---|---|---|---|---|
| 297.5 | 3.61(−6) | 2.86(−5) | 9.56(−7) | 5.47(−8) |
| 300.0 | 3.05(−5) | 1.50(−4) | 8.27(−6) | 4.17(−7) |
| 302.5 | 1.54(−4) | 5.33(−4) | 4.47(−5) | 2.62(−6) |
| 305.0 | 5.24(−4) | 1.39(−3) | 1.63(−4) | 1.34(−5) |
| 307.0 | 1.32(−3) | 2.89(−3) | 4.39(−4) | 5.14(−5) |
| 310.0 | 2.66(−3) | 5.05(−3) | 9.32(−4) | 1.49(−4) |
| 312.5 | 4.53(−3) | 7.75(−3) | 1.66(−3) | 3.43(−4) |
| 315.0 | 6.82(−3) | 1.08(−2) | 2.58(−3) | 6.52(−4) |
| 317.5 | 9.34(−3) | 1.40(−2) | 3.64(−3) | 1.07(−3) |
| 320.0 | 1.19(−2) | 1.71(−2) | 4.76(−3) | 1.57(−3) |
| 323.1 | 2.25(−2) | 3.12(−2) | 9.19(−3) | 3.39(−3) |
| 330.0 | 8.26(−2) | 1.10(−1) | 3.46(−2) | 1.45(−2) |
| 340.0 | 1.09(−1) | 1.40(−1) | 4.71(−2) | 2.12(−2) |
| 350.0 | 1.22(−2) | 1.57(−1) | 5.43(−2) | 2.53(−2) |
| 360.0 | 1.36(−1) | 1.74(−1) | 6.18(−2) | 2.96(−2) |
| 370.0 | 1.47(−1) | 1.86(−1) | 6.76(−2) | 3.30(−2) |
| 380.0 | 1.57(−1) | 1.99(−1) | 7.37(−2) | 3.65(−2) |
| 390.0 | 1.46(−1) | 1.87(−1) | 6.89(−2) | 3.49(−2) |

TABLE 6—$L_\lambda$ VALUES FOR LATITUDE 50° N.[1] [2] [3]—Continued

| λcenter (nm) | Spring | Summer | Fall | Winter |
|---|---|---|---|---|
| 400.0 | 2.12(−1) | 2.69(−1) | 9.90(−2) | 4.98(−2) |
| 410.0 | 2.80(−1) | 3.55(−1) | 1.31(−1) | 6.54(−2) |
| 420.0 | 2.89(−1) | 3.66(−1) | 1.35(−1) | 6.71(−2) |
| 430.0 | 2.79(−1) | 3.52(−1) | 1.31(−1) | 6.47(−2) |
| 440.0 | 3.31(−1) | 4.17(−1) | 1.55(−1) | 7.66(−2) |
| 450.0 | 3.73(−1) | 4.69(−1) | 1.75(−1) | 8.63(−2) |
| 460.0 | 3.78(−1) | 4.75(−1) | 1.78(−1) | 8.74(−2) |
| 470.0 | 3.90(−1) | 4.91(−1) | 1.84(−1) | 8.95(−2) |
| 480.0 | 4.00(−1) | 5.03(−1) | 1.89(−1) | 9.15(−2) |
| 490.0 | 3.78(−1) | 4.76(−1) | 1.79(−1) | 8.62(−2) |
| 500.0 | 3.86(−1) | 4.85(−1) | 1.83(−1) | 8.77(−2) |
| 525.0 | 1.01 | 1.28 | 4.84(−1) | 2.28(−1) |
| 550.0 | 1.05 | 1.33 | 5.03(−1) | 2.32(−1) |
| 575.0 | 1.05 | 1.34 | 5.04(−1) | 2.28(−1) |
| 600.0 | 1.06 | 1.35 | 5.13(−1) | 2.32(−1) |
| 625.0 | 1.08 | 1.37 | 5.26(−1) | 2.42(−1) |
| 650.0 | 1.10 | 1.38 | 5.39(−1) | 2.53(−1) |
| 675.0 | 1.11 | 1.39 | 5.47(−1) | 2.61(−1) |
| 700.0 | 1.11 | 1.38 | 5.49(−1) | 2.66(−1) |
| 750.0 | 2.15 | 2.86 | 2.07 | 5.22(−1) |
| 800.0 | 2.08 | 2.57 | 1.04 | 5.11(−1) |

[1] Units of $L_\lambda$ are $10^{-3}$ einsteins $cm^{-2}$ $day^{-1}$. Multiplication of $L_\lambda$ by $\epsilon_\lambda$ in the units of $molar^{-1}$ $cm^{-1}$ gives the rate constant in units of $day^{-1}$.

[2] The second number in the columns in parenthesis is the power of ten by which the first number is multiplied.

[3] Based on the GC SOLAR program.

(4) *Examples of application of methodology*—(i) *Tier 1 Test: UV-visible absorption spectra—estimation of aqueous photolysis rate constant and minimum half-life in sunlight*—(A) *Illustrative example 1.* A neutral organic chemical A was dissolved in water at a concentration of $1.00 \times 10^{-3}$ M. UV-visible absorption spectra were obtained in a 10.0 cm quartz absorption cell and no absorbance was detected above the baseline in the region 290 nm and greater (i.e., $A_\lambda = 0$) for $\lambda > 290$ nm). Since $A_\lambda = 0$, then $\epsilon_\lambda^c = 0$ (Equation 15). Using this result in Equation 7, it is found that $(k_{pE})_{max.} = 0$, indicating that no direct photolysis can take place in sunlight at any latitude or season of the year. This example illustrates the principle of the Grotthus-Draper law, the first law of photochemistry. That is, in order for direct photolysis to take place in sunlight, the chemical must absorb sunlight in the region $\lambda > 290$ nm.

(B) *Illustrative example 2.* (*1*) Consider a plant located in Columbus, Georgia on the Chattahoochee River which produces an organic chemical B which is not an acid or a base. The waste effluent passes through a primary and secondary treatment plant and is then discharged directly into the river. The plant produces chemical B continuously every day of the year. The plant is located at 32.5° north latitude. Estimate the maximum sunlight direct photolysis rate constant and the corresponding minimum half-life for this chemical in the river for the winter and summer seasons under clear skies.

(*2*) Laboratory experiments, data, and calculations: (*i*) The water solubility of chemical B is $1.00 \times 10^{-3}$ M at 25 °C. Chemical B was dissolved directly in water and a $1.00 \times 10^{-4}$ molar solution was prepared at 25 °C. The UV-visible absorption spectra were obtained according to the Tier 1 procedure in a 10.0 cm quartz absorption cell in duplicate. Using the wavelength interval range (from Table 1 under paragraph (c)(3) of this section), the average absorbance of the duplicate runs at λcenter was obtained and the results are summarized in the following Table 7:

TABLE 7—SUMMARY OF PHOTOLYSIS DATA FOR CHEMICAL B

SPECTRAL DATA

| λ center (nm) | $A_\lambda$ | $\epsilon^c_\lambda$ ($M^{-1}$ $cm^{-1}$) |
|---|---|---|
| 297.5 | 1.684 | 1684 |
| 300.0 | 1.434 | 1434 |
| 302.5 | 1.221 | 1221 |
| 305.0 | 0.919 | 919 |
| 307.5 | 0.742 | 742 |
| 310.0 | 0.206 | 206 |
| 312.5 | 0.138 | 138 |
| 315.0 | 0.094 | 94 |
| 317.5 | 0.057 | 57 |
| 320.0 | 0.009 | 9 |
| 323.1 | 0.002 | 2 |
| 330.0 | 0.000 | 0 |

PHOTOLYSIS DATA

| λ center (nm) | Summer | | Winter | |
|---|---|---|---|---|
| | $L_\lambda$[1] | $\epsilon_\lambda^c L_\lambda$ ($day^{-1}$) | $L_\lambda$[1] | $\epsilon^c_\lambda L_\lambda$ ($day^{-1}$) |
| 297.5 | 1.09(−4) | 0.18 | 6.78(−6) | 0.01 |
| 300.0 | 4.11(−4) | 0.59 | 4.23(−5) | 0.06 |
| 302.5 | 1.14(−3) | 1.39 | 1.71(−4) | 0.21 |
| 305.0 | 2.46(−3) | 2.26 | 4.95(−4) | 0.45 |
| 307.5 | 4.45(−3) | 3.30 | 1.11(−3) | 0.82 |
| 310.0 | 7.02(−3) | 1.46 | 2.04(−3) | 0.42 |
| 312.5 | 1.00(−2) | 1.38 | 3.26(−3) | 0.45 |
| 315.0 | 1.32(−2) | 1.24 | 4.69(−3) | 0.44 |
| 317.5 | 1.64(−2) | 0.94 | 6.21(−3) | 0.35 |
| 320.0 | 1.95(−2) | 0.18 | 7.76(−3) | 0.07 |
| 323.1 | 3.46(−2) | 0.07 | 1.43(−2) | 0.03 |

PHOTOLYSIS DATA—Continued

| λ center (nm) | Summer | | Winter | |
|---|---|---|---|---|
| | $L_\lambda$[1] | $\epsilon_\lambda^{c}L_\lambda$ (day$^{-1}$) | $L_\lambda$[1] | $\epsilon^{c}_\lambda L_\lambda$ (day$^{-1}$) |
| 330.0 | 1.18(−1) | 0.00<br>$\epsilon\lambda^{c}{}_\lambda L_\lambda$ = 12.90 | 5.17(−2) | 0.00<br>$\epsilon\lambda^{c}{}_\lambda L_\lambda$ = 3.31 |

[1] The units of $L_\lambda$ are in $10^{-3}$ einsteins cm $^{-2}$ day $^{-1}$. The second number in the columns in parenthesis is the power of ten by which the first number is multiplied.

(*ii*) From the above data and Equation 15 under paragraph (c)(1)(i)(A) of this section, the average molar absorptivity is

*Equation 18*

$$\epsilon_\lambda = 1000\ A_\lambda.$$

From the average $A_\lambda$ value at $\lambda$ center, the average molar absorptivity can be obtained from Equation 18 and the results are summarized in Table 7 under paragraph (c)(4)(i)(B)(*2*)(*i*) of this section. Since the plant is located at 32.5° north latitude, the closest $L_\lambda$ values are at 30° north latitude. These values are obtained from Table 4 under paragraph (c)(3) of this section and are summarized in Table 8 under paragraph (c)(4)(i)(B)(*2*)(*i*) of this section for the summer and winter seasons. Using the data from Table 7 under paragraph (c)(4)(B)(*2*)(*i*) of this section and Equations 7 and 8 under paragraph (a)(3)(v) of this section, the following results are obtained.

*Summer*

$$(k_{pE})_{max.} = \Sigma\epsilon_\lambda{}^{c}L_\lambda = 13.0\ \text{day}^{-1}$$
$$(t_{1/2E})_{min.} = 0.053\ \text{day}$$

*Winter*

$$(k_{pE})_{max.} = \Sigma\epsilon^{c}L_\lambda = 3.31\ \text{day}^{-1}$$
$$(t_{1/2E})_{min.} = 0.21\ \text{day}$$

Since the chemical transforms rapidly for the summer and winter seasons, it is necessary to carry out Tier 2 experiments to more accurately define direct photolysis rates in aqueous media as a function of the season of the year.

(ii) *Tier 2 Phase 1: Aqueous photolysis in sunlight—illustrative example 3.* (A) Consider the same scenario as described in illustrative example 2, under paragraph (c)(4)(i)(B) of this section. Using the Tier 2, Phase 1 Procedure, carry out experiments to estimate the rate of direct photolysis and half-life in aqueous solution in the spring for water bodies.

(B) Photolysis experiments and calculations: Since chemical B absorbs appreciably below 340 nm, 11 mm i.d. quartz tubes were used (note: this tube has an approximate pathlength of 1 cm). Chemical B was dissolved directly in pure water and a 1.00 x $10^{-5}$ molar solution was prepared at 25°C. Since the water solubility is 1.00 x $10^{-3}$ M at 25°C, this sample solution was well below one-half its water solubility. The uv spectrum of this solution in a one cm absorption cell indicated that $A_\lambda$ was less than 0.05 at 290 nm. Hence, under these conditions, first-order kinetics are applicable.

(C) A series of quartz tubes were filled with this aqueous solution, sealed, and photolysis experiments were carried out in sunlight according to the appropriate procedure described in paragraph (b)(2)(iii)(A) of this section. The experiments were started at noon (1200 hours) on May 8, 1982. The weather conditions are summarized for this period of time and the concentration data given represent the mean of duplicate determinations.

(*1*) May 2, 1982: at t = 0 (noon—1200 hours) $C_o$ = 1.00 x $10^{-5}$ M.

(*2*) May 2, 1982: Noon to sunset—clear and sunny.

(*3*) May 3, 1982: Noon (1200 hours), $C_t$ = 0.840 x $10^{-5}$ M.

(*4*) May 3, 1982: at 1400 hours the weather conditions were cloudy with rain. The rain and cloudy weather continued until 2200 hours.

(*5*) From sunrise, May 4, 1982 through 1200 hours May 8, 1982, the weather was clear and sunny. At 1200 hours, May 8, 1982, analysis of the samples gave an average concentration of $C_t$ = 0.400 x $10^{-5}$ M. Since 60 percent of chemical B transformed, the photolysis experiments were terminated and the control samples were analyzed. The average concentration of the control samples was 0.997 x $10^{-5}$ M which was essentially the same as $C_o$. Hence, no adventitious processes occurred and the loss of chemical was only due to sunlight photolysis.

(D) Listed in the following Table 8 are the times of sunrise and sunset for the dates sunlight photolysis experiments were carried out along with the total number of hours of sunlight.

TABLE 8—SUMMARY OF TIMES FOR SUNRISE AND SUNSET FOR THE PERIOD MAY 2-8, 1982

| Date (1982) | Sunrise (a.m.) | Sunset (p.m. | Total sunlight hours |
|---|---|---|---|
| 5/2 | 0600 | 2010 | 14.2 |
| 5/3 | 0559 | 2011 | 14.2 |
| 5/4 | 0558 | 2012 | 14.2 |
| 5/5 | 0557 | 2013 | 14.3 |
| 5/6 | 0556 | 2014 | 14.3 |
| 5/7 | 0555 | 2015 | 14.3 |
| 5/8 | 0554 | 2016 | 14.4 |

(E) The following data summarizes the dates photolyzed, the times exposed to sunlight, the total sunlight photolysis time for each date in days, the total number of days of sunlight photolysis, and the calculation of $k^c_p$ and $t_{1/2}$.

| Date | Times photolyzed | Sunlight photolysis time for each (days) |
|---|---|---|
| 5/2 | 1200 hrs. to 2010 hrs. (8.2/14.2) | 0.58 |
| 5/3 | 0559 hrs. to 1200 hrs. (6.0/14.2) | 0.42 |
| 5/3 | 1200 hrs. to 1400 hrs. (2.0/14.2) | 0.14 |
| 5/4 | 0558 hrs. to 2012 hrs. | 1.00 |
| 5/5 | 0557 hrs. to 2013 hrs. | 1.00 |
| 5/6 | 0556 hrs. to 2014 hrs. | 1.00 |
| 5/7 | 0555 hrs. to 2015 hrs. | 1.00 |
| 5/8 | 0554 hrs. to 1200 hrs. (6.1/14.4) | 0.42 |

[1] Total hours.

$t=5.6$ days; $C_0=1.00\times10^{-5}$; $C_t=0.400\times10^{-5}$

$\ln(C_0/C_t)=k^c_p t$

$k^c_p=(1/t)\ \ln(C_0/C_t)=\ (1/5.6)\ln(1.00\times10^{-5}/0.400\times10^{-5})$

$k^c_p=0.16\ \text{days}^{-1}$

$t_{1/2}=0.693/0.16\ \text{days}^{-1}=4.3$ days

Therefore, the rate constant for direct photolysis of chemical B in tubes in pure water is 0.16 days$^{-1}$ and the corresponding half-life is 4.3 days for the period of photolysis May 2-8, 1982 at 32.5° north latitude. Using equation 13, under paragraph (b)(2)(i)(J) of this section, the direct photolysis rate constant ($k_{pE}$) for water bodies is 0.073 days$^{-1}$ and the corresponding half-life ($t_{1/2E}$) is 9.5 days.

(iii) *Tier 2, Phase 2: Aqueous photolysis in sunlight—illustrative example* 4. (A) Consider the same scenario as described in illustrative examples 2 and 3. Using the Tier 2, Phase 2, procedure, carry out experiments to determine the sunlight reaction quantum yield and estimate the rate constant for direct photolysis in aqueous solution and the half-life for water bodies and clear sky conditions for the summer and winter seasons.

(B) Photolysis experiments and calculations: The sunlight photolysis experiments were carried out in the beginning of May 1982, at 32.5° north latitude.

(C) Preparation of the actinometer solution: (*1*) The results from the Tier 2, Phase 1, experiments indicated that $K^c_p$ for the test chemical was 0.16 days$^{-1}$. Since the experiments were carried out in early May at 32.5° north latitude, the value of $K^a_a$ was chosen from Table 2 which corresponds to the spring season and at 30° north latitude; and the value is 483 days$^{-1}$. Using Equation 11 under paragraph (b)(2)(i)(H) of this section, the molar concentration of pyridine required to adjust the actinometer rate to match the rate of disappearance of the test chemical is

$[PYR]=26\ .9\ (0.16/483)=8.91\ \times\ 10^{-3}$ molar.

Using this concentration of pyridine, an actinometer solution was (ii)(C) prepared according to the procedure described in paragraph (b)(2) of this section. The quantum yield for this actinometer is calculated using equation 9 under paragraph (a)(3)(vii) of this section.

$\phi^a_E=0.0169[PYR]=0.0169(8.91\ \times\ 10^{-3})=1.51\ \times\ 10^{-4}$

(*2*) Procedures for Tier 2, Phase 2 experiments (under paragraph (b)(2)(iii) of this section) were followed and sunlight experiments were initiated at 1200 hours on May 9, 1982. The mean initial concentration of test chemical was $1.00\ \times\ 10^{-5}$ molar and the mean initial concentration of PNAP was $1.00\ \times\ 10^{-5}$ molar. Samples of the chemical and actinometer and the controls were analyzed in triplicate periodically at 1200 hours on May 10, 11, 13, 15, and 16. On May 16, the photolysis experiments were terminated. The mean concentrations of all samples are summarized as follows:

| Date | Concentration of chemical (M) | Concentration of actinometer (M) | Concentration of chemical control (M) | Concentration of actinometer control (M) |
|---|---|---|---|---|
| May 9 | 1.00 × $10^{-5}$ | 1.00 × $10^{-5}$ | 1.00 × $10^{-5}$ | 1.00 × $10^{-5}$ |
| May 10 | 0.820 × $10^{-5}$ | 0.855 × $10^{-5}$ | 0.997 × $10^{-5}$ | 1.00 × $10^{-5}$ |
| May 11 | 0.654 × $10^{-5}$ | 0.710 × $10^{-5}$ | 1.00 × $10^{-5}$ | 0.997 × $10^{-5}$ |
| May 13 | 0.440 × $10^{-5}$ | 0.515 × $10^{-5}$ | 0.996 × $10^{-5}$ | 0.999 × $10^{-5}$ |
| May 15 | 0.299 × $10^{-5}$ | 0.383 × $10^{-5}$ | 0.999 × $10^{-5}$ | 0.998 × $10^{-5}$ |
| May 16 | 0.233 × $10^{-5}$ | 0.304 × $10^{-5}$ | 0.997 × $10^{-5}$ | 0.996 × $10^{-5}$ |

Since no significant loss of PNAP or test chemical was observed in the control samples, no adventitious processes occurred and the loss of test chemical and PNAP was only due to sunlight photolysis.

(*3*) Using the above data, ln $(C_o/C_t)$ for the test chemical and actinometer can be calculated and the results are summarized as follows:

| t (days) | Chemical | | Actinometer | |
|---|---|---|---|---|
| | $C_t \times 10^5$ (M) | ln $(C_o/C_t)^c$ | $C_t \times 10^5$ (M) | ln $(C_o/C_t)^a$ |
| 0 | 1.00 | 0.000 | 1.00 | 0.000 |
| 1 | 0.820 | 0.199 | 0.855 | 0.157 |
| 2 | 0.654 | 0.425 | 0.710 | 0.343 |
| 4 | 0.440 | 0.821 | 0.515 | 0.664 |
| 6 | 0.299 | 1.21 | 0.383 | 0.960 |
| 7 | 0.233 | 1.46 | 0.304 | 1.19 |

(*4*) The ratio of the rate constants, $k^c_p/k^a_p$, is defined by equation 12 under paragraph (b)(2)(i)(H)(3) of this section.

*Equation 12*

$$\ln(C_o/C_t)^c=(k^c_p/k^a_p)\ \ln(C_o/C_t)^a.$$

(*5*) Using all the data (including the time point t=0) and linear regression analysis, the slope is found to be 1.237 with a correlation coefficient of 0.9998. Therefore,

$$(k^c_p/k^a_p)=1.24.$$

(*6*) Using the molar absorptivities obtained in example 2 under paragraph (c)(4)(i)(B) of this section and the $L_\lambda$ values for spring at 30° north latitude in Table 4 under paragraph (c)(3) of this section, the value of $\sum \epsilon^c_\lambda L_\lambda$ can be calculated as follows:

| λ center (nm) | $\epsilon^c_\lambda (M^{-1}cm^{-1})$ | $L^1_\lambda$ | $\epsilon^c_\lambda L_\lambda$ (days$^{-1}$) |
|---|---|---|---|
| 297.5 | 1684 | 5.73 (−5) | 0.10 |
| 300.0 | 1434 | 2.50 (−4) | 0.36 |
| 302.5 | 1221 | 7.65 (−4) | 0.93 |
| 305.0 | 919 | 1.79 (−3) | 1.65 |
| 307.5 | 742 | 3.43 (−3) | 2.55 |
| 310.0 | 206 | 5.64 (−3) | 1.17 |
| 312.5 | 138 | 8.27 (−3) | 1.14 |
| 315.0 | 94 | 1.12 (−2) | 1.05 |
| 317.5 | 57 | 1.41 (−2) | 0.80 |
| 320.0 | 9 | 1.70 (−2) | 0.15 |
| 323.1 | 2 | 3.04 (−2) | 0.06 |
| 330.0 | 0 | 1.05 (−1) | 0.00 |

[1] The units of $L_\lambda$ are $10^{-3}$ einsteins $cm^{-2}$ $day^{-1}$.

$\Sigma\epsilon^c_\lambda L_\lambda=9.96$ days$^{-1}$.

For this experiment, $k^a_a(\Sigma=\epsilon^a_\lambda L_\lambda)$ is 483 days$^{-1}$ (Table 2 under paragraph (c)(3) of this section). All the pertinent data are summarized as follows:

$k^c_p/k^a_p=1.24$; $\Sigma\epsilon^c_\lambda L_\lambda=9.96$ days$^{-1}$; $\Sigma\epsilon^a_\lambda L_\lambda=483$ days$^{-1}$; $\phi^a_E=1.51\times10^{-4}$.

Substituting these results into Equation 10 under paragraph (a)(3)(vii) of this section yields

$$\phi^c_E=(1.24)(483/9.96)(1.51\times10^{-4})$$
$$\phi^c_E=9.08\times10^{-3}.$$

(*7*) The rate constants for direct photolysis of test chemical in aqueous media and the half-life for water bodies and clear sky conditions for the winter and summer seasons can be calculated as follows: The values of $\sum\epsilon_\lambda{}^c L_\lambda$ have been calculated from example under paragraph (c)(3)(i)(A) of this section. For summer $\sum\epsilon_\lambda{}^c L_\lambda=13.0$ days$^{-1}$; for winter $\Sigma\epsilon_\lambda{}^c L_\lambda=3.31$ days$^{-1}$. The reaction quantum yield for the chemical is 9.08 × $10^{-3}$. Using these data in equation 4 under paragraph (a)(3)(iii) of this section yields

*Summer*

$$k_{pE}=9.08\times10^{-3}(13.0)=0.118\ \text{days}^{-1}$$

*Winter*

$$k_{pE}=9.08\times10^{-3}(3.31)=0.0301\ \text{days}^{-1}.$$

These values can be substituted into Equation 2 under paragraph (a)(3)(ii) of this section to obtain the half-lives for these two seasons.

*Summer:*

$t_{1/2E}=(0.693/0.118)=5.9$ days

*Winter:*

$t_{1/2E}=(0.693/0.0301)=23$ days.

(5) *Glossary of important symbols*

PYR—Pyridine.
PNAP—p-Nitroacetophenone.
$\lambda$—Wavelength $\lambda$.
$A_\lambda$—Absorbance at wavelength $\lambda$.
a—Actinometer (composed of PNAP/PYR).
$\epsilon^c_\lambda$—Molar absorptivity of a chemical C.
$\epsilon^a_\lambda$—Molar absorptivity of the actinometer.
l—light pathlength; the distance traveled by a beam of light passing through the system.
$\phi^c_E$—Sunlight reaction quantum yield of chemical c in water.
$\phi^a_E$—Sunlight reaction quantum yield of the actinometer in water. Since the reaction quantum yield is independent of $\lambda$, $\phi^a_E=\phi^a$ (i.e., the reaction quantum yield of the actinometer measured in the laboratory).
[C]—Molar concentration of chemical c.
[PYR]—Molar concentration of pyridine.
$-d[C]/dt$—Direct photolysis rate of chemical c.
$k_{pE}$—Direct photolysis sunlight rate constant in water bodies in the environment.
$(k_{pE})_{max.}$—Maximum direct photolysis sunlight rate constant in water bodies in the environment.
$k^c_p$—Direct photolysis sunlight rate constant of chemical c in water in tubes.
$k^a_p$—Direct photolysis sunlight rate constant of the actinometer in water in tubes.
$k_{a\lambda}$—Specific light absorption of a photoreactive chemical at a low concentration and at wavelength $\lambda$.
$k_a$—Specific light absorption rate constant integrated over all wavelengths absorbed by the chemical.
$k^a_a$—Specific light absorption rate constant integrated over all wavelengths absorbed by the actinometer.
$t_{1/2}$—Sunlight half-life of a chemical in water in tubes.
$(t_{1/2E})_{min.}$—The minimum sunlight half-life of a chemical in water bodies in the environment.
I—The numbers of photons of light of wavelength $\lambda$ in the system per cm$^2$ per second.
$L_\lambda$—Solar irradiance in water in the units $10^{-3}$ einsteins cm$^{-2}$ day$^{-1}$.
$\gamma$—The geometry factor which represents the ratio of the rate constants in tubes ($k_p$) to the rate constant in water bodies in the environment ($k_{pE}$).

(d) *References.* For additional background information on this test guideline the following references should be consulted:

(1) *Astronomical Almanac* (1982).

(2) Dulin, D. and Mill, T. "Development and application of solar actinometers." *Environmental Science and Technology* 16:815 (1982).

(3) *Handbook of Chemistry and Physics.* (Chemical Rubber Co., Cleveland, Ohio)

(4) Mill, T., Davenport, S.E., Dulin D.E., Mabey, W.R., and Bawol, R. *Evaluation and Optimization of Photolysis Screening Protocols.* EPA Report No. 560/5-81-003 (1981).

(5) Mill, T., Mabey, W.R., Bomberger, D.C., Chou T-W., Hendry, D.G., and Smith, J.H. *Laboratory Protocols for Evaluating the Fate of Organic Chemicals in Air and Water.* EPA Report No. 600/3-82-022 (1982).

(6) Mill, T., Mabey, W.R., Hendry, D.G. Winterle, J., Davenport, J., Barich, V., Dulin, D., and Tse, D. *Design and Validation of Screening and Detailed Methods for Environmental Processes.*

(7) U.S. Environmental Protection Agency, Mill, T., Davenport, J.E., Winterle, J.S., Mabey, W.R., Drossman, H., Tse, D., and Liu, A. "Toxic substances process data generation and protocol development," Draft final report, EPA Contract No. 68-03-2981 with EPA Athens Research Laboratory, Office of Research and Development (1984).

(8) Zepp, R.G., and Cline, D.M. "Rates of direct photolysis in aquatic environment" *Environmental Science and Technology* 11:359 (1977).

(9) Zepp, R.G."Quantum yields for reaction of pollutants in dilute aqueous solution" *Environmental Science and Technology,* 12:327 (1978).

(10) Zepp, R.G. Environmental Research Laboratory, U.S. Environmental Protection Agency, College Station Road, Athens, Georgia 30601.

[50 FR 39252, Sept. 27, 1985, as amended at 53 FR 10391, Mar. 31, 1988; 53 FR 12526, Apr. 15, 1988]

## § 796.3780 Laboratory determination of the direct photolysis reaction quantum yield in aqueous solution and sunlight photolysis.

(a) *Introduction*—(1) *Background and purpose.* (i) Numerous chemicals enter natural aquatic water bodies from a variety of sources. Some pollutants present in aqueous media can undergo photochemical transformation in sunlight by direct photolysis. Therefore, quantitative data in the form of rate constants and half-lives are needed to determine the importance of direct photochemical transformation of pollutants in aqueous media.

(ii) Section 796.3700, the first in a series of aqueous photolysis test methods, was designed to determine the molar absorptivity and reaction quantum yield of a test chemical in aqueous solution. These parameters can be combined with solar irradiance data to determine environmentally relevant rate constants and half-lives in aqueous solutions as a function of latitude and season of the year anywhere in the United States.

(iii) Section 796.3700 was developed as a screening test to obtain the direct photolysis reaction quantum yield of a chemical in aqueous solution by carrying out photolysis experiments in sunlight. This method does not require sophisticated and expensive photochemical equipment and therefore the reaction quantum yield can be determined easily and at a modest cost. However, there are circumstances when this method may not be applicable. For example, this procedure is not applicable for determining the reaction quantum yield of a test chemical when the temperature outdoors falls below 0° C. Furthermore, depending upon the status of a risk assessment for a specific chemical, a more precise value of the reaction quantum yield may be required. Thus, a more comprehensive procedure is needed to determine the direct photolysis reaction quantum yield in the laboratory using specialized photochemical equipment and monochromatic (or narrow band) light. This section describes laboratory procedures for determining the direct photolysis reaction quantum yield in aqueous solution.

(iv) The reaction quantum yield obtained in this test method can be combined with molar absorptivity data and solar irradiance data to determine environmentally relevant rate constants and half-lives in aqueous solution as a function of latitude and season of the year anywhere in the United States.

(v) The procedures described in this test method are very detailed, and the theory of photolysis in aqueous solution is relatively complicated. In order to follow these procedures, it is recommended that the Technical Support Document in the public record for this section should first be studied carefully.

(2) *Definitions and units.* (i) "Radiant energy", or radiation, is defined as the energy traveling as a wave unaccompanied by transfer of matter. Examples include x-rays, visible light, ultraviolet light, radio waves, etc.

(ii) "Absorbance ($A_\lambda$)" is defined as the logarithm to the base 10 of the ratio of the initial intensity ($I_o$) of a beam of radiant energy to the intensity (I) of the same beam after passage through a sample at a fixed wavelength λ. Thus, $A_\lambda = \log_{10} (I_o/I)$.

(iii) The "Beer-Lambert law" states that the absorbance of a solution of a given chemical species, at a fixed wavelength, is proportional to the thickness of the solution (l), or the light pathlength, and the concentration of the absorbing species (C).

(iv) "Molar absorptivity ($\epsilon_\lambda$)" is defined as the proportionality constant in the Beer-Lambert law when the concentration is given in terms of moles per liter (i.e., molar concentration). Thus, $A_\lambda = \epsilon_\lambda Cl$, where $A_\lambda$ and $\epsilon_\lambda$ represent the absorbance and molar absorptivity at wavelength λ and l and C are defined in paragraph (a)(2)(iii) of this section. The units of $\epsilon_\lambda$ are molar $^{-1}$ cm$^{-1}$ Numerical values of molar absorptivity depend upon the nature of the absorbing species.

(v) A "first-order reaction" is defined as a reaction in which the rate of disappearance of a chemical is directly proportional to the concentration of the chemical and is not a function of the concentration of any other chemical present in the reaction mixture.

(vi) A "zero-order reaction" is defined as a reaction in which the rate of disappearance of a chemical is independent of the concentration of the chemical or the concentration of any other chemical present in the reaction mixture.

(vii) The "first-order half-life ($t_{1/2}$)" of a chemical is defined as the time required for the concentration of the chemical to be reduced to one-half its initial value.

(viii) The "sunlight direct aqueous photolysis rate constant ($k_{pE}$)" is the first-order direct photolysis rate constant in the units of day$^{-1}$ and is a measure of the rate of disappearance of a chemical dissolved in a water body in sunlight.

(ix) The "solar irradiance in water ($L_\lambda$)" is related to the sunlight intensity in water at shallow depths and is proportional to the average light flux (in the units of $10^{-3}$ einsteins cm$^{-2}$ day$^{-1}$) that is available to cause photoreaction in the wavelength interval centered at λ over a 24-hour day at a specific latitude and season date.

(x) "The Grotthus-Draper law," the first law of photochemistry, states that only light which is absorbed can be effective in producing a chemical transformation.

(xi) The "Stark-Einstein law," the second law of photochemistry, states that only one molecule is activated to an excited state per photon or quantum of light absorbed.

(xii) The "reaction quantum yield ($\phi_\lambda$)" for an excited state process is defined as the fraction of absorbed light that results in photoreaction at a fixed wavelength λ. It is the ratio of the number of molecules that photoreact to the number of quanta of light absorbed or the ratio of the number of moles that photoreact to the number of einsteins of light absorbed at a fixed wavelength λ.

(xiii) "Direct photolysis" is defined as the direct absorption of light by a chemical followed by a reaction which transforms the parent chemical into one or more products.

A glossary of symbols can be found under paragraph (c)(4) of this section.

(3) *Principle of the test method.* (i) This test method is based on the principles developed by Zepp (1978) under paragraph (d)(13) of this section, the use of low optical density actinometers developed by Mill et al. (1981, 1982) under paragraphs (d) (7) and (8) of this section , and Dulin and Mill (1982) under paragraph (d)(4) of this section, and the high optical density ferrioxalate actinometer developed by Parker (1953) under paragraph (d)(12) of this section, and Hatchard and Parker (1956) under paragraph (d)(6) of this section.

(ii) Zepp (1978), under paragraph (d)(13) of this section, published a paper on the determination of the reaction quantum yield for the reaction of pollutants in dilute aqueous solution in the laboratory. Based on this work, two procedures are described to determine the reaction quantum yield of a test chemical at low optical density in the laboratory. These procedures involve the use of: low-optical density test chemical and actinometer; and low-optical density test chemical and high-optical density actinometer.

(A) *Procedure One: Determination of the Reaction Quantum Yield by the Low-Optical Density Test Chemical and Actinometer Method.* (*1*) For a low optical density test chemical and actinometer in which the absorbance of aqueous solutions is less than 0.02, the reaction quantum yield of a test chemical $\theta_c$ at wavelength λ is given by equation

*Equation 1*

$$\phi_c=(k_{pc}/k_{pa})(\epsilon_{\lambda a}/\epsilon_{\lambda c})\phi_a$$

where $\epsilon_a$ and $\epsilon_{\lambda c}$ are molar absorptivities of the actinometer and test chemical, respectively, at wavelength λ, $\phi_a$ is the reaction quantum yield of the actinometer at wavelength λ, and $k_{pa}$ and $k_{pc}$ are the first-order direct photolysis rate constant for actinometer and test chemical, respectively. These rate constants are defined by the equations

*Equation 2*

$$\ln(C_o/C_t)_a=k_{pa}t$$

*Equation 3*

$$\ln(C_o/C_t)_c=k_{pc}t$$

where $(C_o)_a$ and $(C_t)_a$ are the molar concentrations of actinometer at time t=0 and t and $(C_o)_c$ and $(C_t)_c$ are the molar concentrations of test chemical at time t=0 and t. Since $k_{pa}$ and $k_{pc}$ are first-order rate constants, the half-lives of actinometer and test chemical are:

*Equation 4*

$$(t_{1/2})_a=0.693/k_{pa}$$

*Equation 5*

$(t_{½})_c = 0.693/k_{pc}$.

(*2*) If both the actinometer and test chemical solutions are photolyzed in identical cells, equation 3 under paragraph (a)(3)(ii)(A)(*1*) of this section can be divided into equation 2 under paragraph (a)(3)(ii)(A)(*1*) of this section. Carrying out this operation and rearranging the resultant equation yields

*Equation 6*

$\ln(C_o/C_t)_c = (k_{pc}/k_{pa})\ln(C_o/C_t)_a$.

(*3*) Procedure One involves the simultaneous photolysis of test chemical and actinometer in an Ace-type photochemical "merry-go-round" reactor (PMGRR) using monochromatic light of wavelength λ. Two low optical density actinometers have been developed by SRI International for the Office of Toxic Substances/U.S. Environmental Protection Agency to measure the light intensity incident on the sample during photolysis [Mill et al. (1981, 1982) under paragraphs (d) (7) and (8) of this section and Dulin and Mill (1982) under paragraph (d)(4) of this section]. These actinometers are:

(*i*) p-nitroacetophenone-pyridine actinometer (PNAP/PYR); and

(*ii*) p-nitroanisole-pyridine actinometer (PNA/PYR). The rate constant of each of these actinometers can be adjusted to match the rate constant of the test chemical by adjusting the concentration of pyridine. Since the rate constant is a function of the reaction quantum yield of the actinometer, the rate constant can be adjusted according to the following equations:

*Equation 7*

PNAP/PYR actinometer $\phi_a = 0.0169[PYR]$

*Equation 8*

PNA/PYR actinometer $\phi_a = 0.437[PYR] + 0.000282$,

where [PYR] is the molar concentration of pyridine for a PNAP or PNA concentration of approximately 1 x $10^{-5}$ M.

(*4*) The laboratory procedure for determining the reaction quantum yield of a test chemical in aqueous solution has been divided into three phases using a UV-visible absorption spectrophotometer, an Ace-type PMGRR, and a 450 watt medium pressure mercury lamp with appropriate filters to isolate the monochromatic wavelength λ. In Phase 1, the molar absorptivities of a test chemical, $\epsilon_{313_c}$ and $\epsilon_{366_c}$ are determined with a UV-visible absorption spectrophotometer using procedures outlined in §§796.1050 and 796.3700. Based on these results, photolysis experiments are carried out at 313 or 366 nm corresponding to the higher value of $\epsilon_{313_c}$ or $\epsilon_{366_c}$.

(*5*) The Phase 2 procedure is composed of trial photolysis experiments at the chosen wavelength λ (313 or 366 nm) in an Ace-type PMGRR to determine the approximate rate constant and half-life of the test chemical and to choose the appropriate actinometer which has a rate constant approximately the same as the rate constant of the test chemical. First, an aqueous solution of test chemical at low optical density is photolyzed in the PMGRR at the chosen wavelength λ to determine $k_{pc}$ and $(t_{½})_c$ using equations 3 and 5, respectively under paragraph (a)(3)(ii)(A)(*1*) of this section. If $(t_{1/2})_c$ is less than 12 hours, use the PNA/PYR actinometer. If $(t_{1/2})_c$ is greater than 12 hours, then use the PNAP/PYR actinometer. Trial photolysis experiments are then carried out at wavelength λ with the chosen low optical density actinometer to determine the molar concentration of pyridine needed to make the rate constant of the actinometer approximately match the rate constant of the test chemical.

(*6*) In the Phase 3 procedure, low-optical density aqueous solutions of test chemical and actinometer (at a fixed molar concentration of pyridine [PYR]) are photolyzed in identical tubes in the PMGRR at wavelength λ. Concentrations of test chemical and actinometer are measured as a function of time. These data are used in Equation 6 under paragraph (a)(3)(ii)(A)(*2*) of this section to determine the ratio of the rate constants $(k_{pc}/k_{pa})$. The reaction quantum yield of the actinometer $\phi_a$, employed in these experiments, can be determined at the molar concentration of pyridine [PYR] using Equation 7 or 8 under paragraph (a)(3)(ii)(A)(*3*) of this section. These data, along with the molar absorptivity of the test chemical ($\epsilon_{313c}$ or $\epsilon_{366c}$) and the actinometer ($\epsilon_{313a}$ or $\epsilon_{366a}$) are substituted in equation 1 under paragraph (a)(3)(ii)(A)(*1*) of this section to determine the reaction quantum yield of the test chemical $\phi_c$.

(B) *Procedure Two: Determination of the reaction quantum yield by the low-optical density test chemical and high optical density actinometer method.* (*1*) For a low-optical density test chemical (absorbance <0.02) and for a high optical density actinometer (absorbance >2), such as the ferrioxalate actinometer, the reaction quantum yield of the test chemical $\phi_c$ at wavelength λ is given by the equation

*Equation 9*

$$\phi_c=\phi_a(k_{pc}/k_{pa})(2.303\epsilon_{\lambda c}l)^{-1},$$

where $\phi_a$ is the reaction quantum yield of the ferrioxalate actinometer at wavelength λ, $\epsilon_{\lambda c}$ is the molar absorptivity of the test chemical at wavelength λ, l is the cell pathlength, $k_{pc}$ is the first-order direct photolysis rate constant for the test chemical, and $k_{pa}$ is the zero-order direct photolysis rate constant for the ferrioxalate actinometer. These rate constants are defined by the equations.

*Equation 10*

$$\ln(C_o/C_t)_c=k_{pc}t$$

*Equation 11*

$$(C_t)_a=k_{pa}t,$$

where $(C_o)_c$ and $(C_t)_c$ are the molar concentrations of the test chemical at time t=o and t and $(C_t)_a$ is the molar concentration of the ferrous ion formed at time t.

(*2*) The laboratory procedure for determining the reaction quantum yield of the test chemical is divided into two phases using a UV-visible absorption spectrophotometer and a photochemical optical bench (POB) or a PMGRR containing a 450-watt medium pressure lamp with appropriate filters to isolate the monochromatic wavelength λ. In Phase 1, the molar absorptivities of test chemical ($\epsilon_{313c}$ and $\epsilon_{366c}$) are determined using spectroscopic procedures outlined in §§ 796.1050 and 796.3700. Based on these results, photolysis experiments are carried out at 313 or 366 nm corresponding to the higher value of $\epsilon_{313c}$ or $\epsilon_{366c}$.

(*3*) In the Phase 2 procedure, aqueous low-optical density test chemical solution (absorbance <0.02) and aqueous high-optical density solution of ferrioxalate actinometer (absorbance >2) are photolyzed sequentially in identical cells in POB or PMGRR at wavelength λ. In the first and third set of experiments, the ferrioxalate actinometer is photolyzed for a few minutes and the molar concentration of ferrous ion formed $(C_t)_a$ is measured as a function of time t. These data are used in Equation 11 under paragraph (a)(3)(ii)(B)(*1*) of this section to determine an average value of the actinometer rate constant $(k_{pa})_{ave.}$. The second series of experiments involves the photolysis of the aqueous solution of test chemical in the POB or PMGRR in identical cells to those used in the actinometer experiments and the molar concentration of test chemical $(C_t)_c$ is measured as a function of the time t. These data are used in Equation 10 under paragraph (a)(3)(ii)(B)(*1*) of this section to determine $k_{pc}$. Using $k_{pc}$ and $(k_{pa})_{ave.}$, the molar absorptivity of the test chemical ($\epsilon_{313c}$ or $\epsilon_{366c}$) , the pathlength of the cell l, and the reaction quantum yield of the ferrioxalate actinometer $\phi_a$ at wavelength λ in Equation 9 under paragraph (a)(3)(ii)(B)(*1*) of this section, the reaction quantum yield of the test chemical $\phi_c$ can be determined.

(*4*) As described in detail in § 796.3700 and the public record for that test guideline, and briefly in the public record for this section, the direct sunlight photolysis of a chemical in an optically thin aqueous solution can be described by the following equations:

*Equation 12*

$$\ln(C_o/C_t)=k_{pE}t$$

*Equation 13*

$$t_{1/2E}=0.693/k_{pE}$$

*Equation 14*

$$k_{pE}=\phi_E k_a.$$

where $\phi_E$ is the reaction quantum yield of the chemical in dilute solution and is independent of the wavelength, $k_a=\Sigma k_{a\lambda}$, the sum of $k_{a\lambda}$ values for all wavelengths of sunlight that are absorbed by the chemical (i.e., the light absorption rate constant), t is the time, $C_o$ and $C_t$ are the molar concentrations of chemical at t=0 and t, and $t_{1/2E}$ represents the half-life. The term $k_{pE}$ represents the first-order direct photolysis rate constant for a chemical in a water body in sunlight in the units of reciprocal time. In general, the reaction quantum yield $\phi_E$ is equivalent to the reaction quantum yield $\phi_c$ determined in the laboratory.

(*5*) Furthermore, under the same conditions cited above, the first-order direct photolysis rate constant, $k_{pE}$, is given by the equation.

*Equation 15*

$$k_{pE}=\phi_E\Sigma\epsilon_{\lambda_c}L_\lambda,$$

where $\phi_E$ is the reaction quantum yield, $\epsilon_{\lambda_c}$ is the molar absorptivity in the units molar$^{-1}$ cm$^{-1}$, $L_\lambda$ is the solar irradiance in water in the units $10^{-3}$ einsteins cm$^{-2}$ day$^{-1}$, and the summation is taken over the range $\lambda=290$ to 800 nm. $L_\lambda$ is the solar irradiance at shallow depths for a water body under clear sky conditions and is a function of latitude and season of the year. Solar irradiance data are tabulated in § 796.3700(c)(3), Tables 3-6 as a function of latitude and season of the year.

(*6*) A simple screening test has been developed in § 796.3700(c)(1)(i)(A) Equation 15. As an approximation, it has been assumed that the reaction quantum yield $\phi_E$ is equal to one, the maximum value. As a result, the upper limit for the direct photolysis sunlight rate constant in aqueous solution is obtained and Equation 15 becomes

*Equation 16*

$$(k_{pE})_{max.}=\Sigma\epsilon_{\lambda_c}L_\lambda.$$

Using Equation 16 in Equation 13 under paragraph (a)(3)(ii)(B)(4) of this section, the lower limit for the half-life is then given by

*Equation 17*

$$(t_{1/2E})_{min.}=0.693/(k_{pE})_{max.}.$$

The molar absorptivity can be determined experimentally by the method outlined in paragraph (b)(2)(i) of this section and the solar irradiance data are tabulated in § 796 (c)(3), Tables 3 to 6. These data can then be used in Equation 16 to calculate $(k_{pE})_{max.}$. Finally, $(k_{pE})_{max.}$ can be substituted in Equation 17 to calculate $(t_{1/2E})_{min.}$. Based on these data, a decision can be made whether to determine the reaction quantum yield of the test chemical in the laboratory.

(*7*) Once the reaction quantum yield has been determined in the laboratory by Procedures One or Two under paragraphs (b) (2) and (3) of this section, it can be combined with the molar absorptivity date $\epsilon_{\lambda_c}$ and the appropriate $L_\lambda$ values to calculate $k_{pe}$ as a function of latitude and season of the year anywhere in the United States, using Equation 15 under paragraph (a)(3)(ii)(B)(*5*) of this section. The corresponding half-life can be calculated using $k_{pe}$ in Equation 13 under paragraph (a)(3)(ii)(B)(*4*) of this section.

(4) *Applicability and specificity.* (i) For environmental photochemistry, the general procedures outlined in this test method are applicable to all chemicals which have UV-visible absorption maxima in the range 290 to 800 nm. Some chemicals have absorption maxima significantly below 290 nm and consequently cannot undergo direct photolysis in sunlight (e.g., chemicals such as alkanes, alkenes, alkynes, saturated alcohols, and saturated acids). This is a direct consequence of the Grotthus-Draper law of photochemistry. Some chemicals have absorption maxima significantly below 290 nm but have measurable absorption tails above the baseline in their absorption spectrum at wavelengths greater than 290 nm. Photolysis experiments should be carried out for these chemicals.

(ii) These test methods are applicable to pure chemicals and not to technical grade chemicals.

(iii) The molar absorptivity data ($\epsilon_{\lambda_c}$) obtained in paragraph (b)(2)(i) of this section can be combined with the appropriate $L_\lambda$ data to estimate $(k_{pE})_{max.}$ and $(t_{1/2E})_{min.}$ at shallow depths in water bodies as a function of latitude and season of the year in the U.S. If these data indicate that aqueous photolysis is an important process relative to other transformation processes (e.g., biodegradation, hydrolysis, oxidation, etc.), then it is recommended that the reaction quantum yield $\phi_c$ be determined in the laboratory by Procedures

One or Two outlined under paragraphs (b) (2) and (3) of this section. Once $\phi_c$ has been determined, it can be combined with the molar absorptivity data $\epsilon_{\lambda c}$ and the appropriate $L_\lambda$ data to calculate $k_{pE}$ at shallow depths in water bodies as a function of latitude and season of the year in the United States. The corresponding half-life can then be calculated from $k_{pE}$.

(iv) Procedure One under paragraph (b)(2) of this section is only applicable to solutions of test chemicals and actinometers which have low optical densities (i.e., absorbance $<0.02$). Procedure Two under paragraph (b)(3) of this section is only applicable to solutions of test chemicals with low optical densities and actinometers with high optical densities (i.e., absorbance $>2$).

(v) Procedure One, as described in detail under paragraph (b)(2) of this section, is limited to the spectral region 290 to 400 nm because the recommended low optical density actinometers are only sensitive to light in this region. However, Procedure Two, under paragraph (b)(3) of this section using the ferrioxalate actinometer is useful in the spectral region 290 to 500 nm. This procedure can be extended up to approximately 750 nm using the Reinicke's salt actinometer [de Mayo and Shizuka (1976) under paragraph (d)(3) of this section].

(vi) There is a third procedure which could be used to determine $\phi_c$ in the laboratory. This procedure involves the determination of $\phi_c$ under the conditions when the test chemical and actinometer solutions both have high optical density. However, in general, this method is limited to only very soluble or strongly absorbing organic compounds. Thus, this method is not described in detail as a procedure in this test method. However, this method is briefly described in the public record for this section.

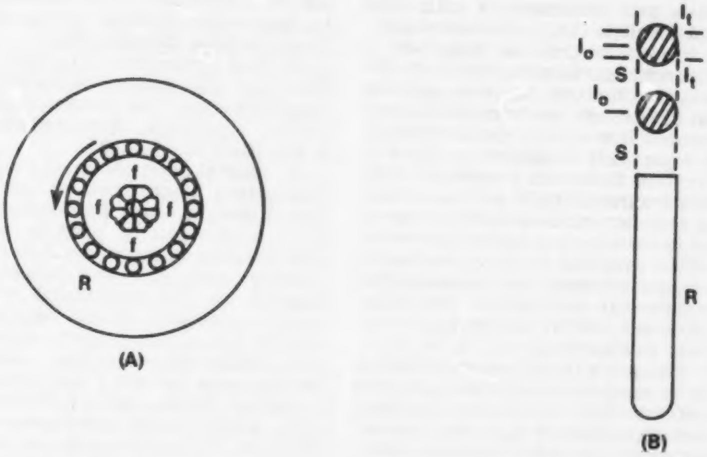
(vii) This test method has been designed to determine the molar absorptivity of a test chemical, $\epsilon_{\lambda c}$, and its quantum yield, $\phi_c$. These parameters can be used to determine environmental rate constants at low absorbance and shallow depths in pure water as a function of latitude and season of the year. Tables 3 to 6 containing solar irradiance data have been included in § 796.3700 to carry out all the calculations. However, the method is really very general and can be extended to determine the rates of photolysis over a range of other environmental conditions using a computer program. Zepp has written a GC SOLAR computer program to calculate the rates of photolysis as a function of depth in water, as a function of the attenuation (or absorption) coefficient of the water ($a\lambda$) for natural water bodies, the average ozone layer thickness that pertains to the seasons and location of interest, and as a function of latitude and season of the year. The computer program is available on written request to R. Zepp, Environmental Research Laboratory, U.S. Environmental Protection Agency, College Station Road, Athens, Georgia 30601.

(b) *Test procedures*—(1) *Test conditions*—(i) *UV-visible absorption spectrophotometer.* The recommended uv-visible absorption spectrophotometer is described in § 796.1050.

(ii) *Special photochemical laboratory Equipment.* There are a number of different designs of photochemical equipment which can be used to measure the reaction quantum yield in the laboratory and the one chosen will depend on the equipment available and to a certain extent on the light source and filter system chosen for the measurement. The apparatus chosen must contain a light source, appropriate filters, sample holders, and cells which allow solutions of test chemical and actinometer to be reproducibly irradiated with a uniform and constant amount of light at a discrete or narrow band wavelength $\lambda$. The temperature of the reaction cells must be at reasonably constant temperature $t_s \pm 2$ °C in the range 20 to 30 °C. The apparatus should be housed in a separate part of a laboratory and properly shielded so that laboratory personnel are not exposed to uv light and to exclude extraneous light that could contribute to the photoreaction of chemicals. If the ferrioxalate actinometer is used, then actinometry experiments must be carried out in a darkroom with photographic "safelights". Excellent reviews and descriptions of a wide variety of photochemical equipment used to measure the reaction quantum yield in the laboratory are given by Calvert and Pitts (1966) under paragraph (d)(2) of this section, de Mayo and Shizuka (1976) under paragraph (d)(3) of this section, Murov (1973) under paragraph (d)(10) of this section, and Mill et al. (1982) under paragraph (d)(9) of this section, and these references are highly recommended.

(A) *Design of the apparatus.* (*1*) *Photochemical "Merry-Go-Round" Reactor.* (*i*) The design of the photochemical "merry-go-round" reactor (PMGRR) has been described in the literature [Moses et al. (1969) under paragraph (d)(11) of this section, Murov (1973) under paragraph (d)(10) of this section, de Mayo and Shizuka (1976) under paragraph (d)(3) of this section]. In the design of a PMGRR, the light source is in the center of the apparatus with reaction tubes arranged in a ring around the light source. The ring rotates around the light source to give a uniform irradiation of the reaction tubes, as depicted in the following Figure 1:

FIGURE 1—"MERRY-GO-ROUND" REACTOR



**(A) "MERRY-GO-ROUND" APPARATUS**

**(B) CYLINDRICAL CELL (R). $I_o$ = INCIDENT LIGHT; $I_t$ = TRANSMITTED LIGHT PASSED THROUGH CELL; S = SLIT**

(A) "Merry-go-round" Apparatus;

(B) Cylindrical cell (R). $I_o$=incident light;

$I_t$=transmitted light passed through cell;

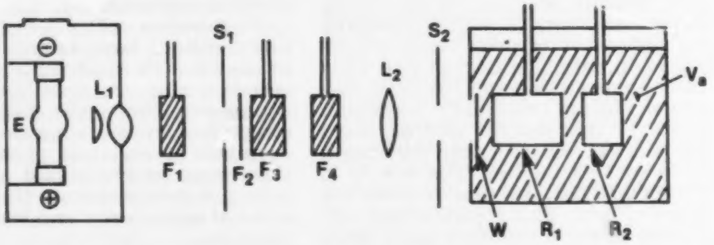
S=slit.

Glass filters (f) may be inserted between the light source and the reaction tubes. Filter solutions (f) may also be contained in the immersion well holding the light source or in a glass donut that surrounds the light source. In order to dissipate the heat generated by the light source, the PMGRR may be immersed in a water bath or a stream of air may be passed through the space between the light source and the filters. The filter solutions can be circulated to an external cooling source. There are two types of PMGRR equipment; the Ace-type and the Moses-type and both are very similar. The Ace-type PMGRR is designed so that the entire reaction cell is irradiated while the Moses-type PMGRR [Moses et al. (1969) under paragraph (d)(11) of this section] is designed with windows (which act as slits with a fixed aperture) so that only a narrow portion of the reaction cell is irradiated. Since the Ace-type PMGRR is the only one commercially available, this test method has been specifically designed to use this PMGRR. However, if the Moses-type PMGRR is available, this test method has to be slightly modified as described in public record for this section.

(*ii*) Small cylindrical tubes are used in the PMGRR to hold small volumes of test chemical and actinometer solutions. One tube is used for each datum point measured.

(2) *Photochemical optical bench.* The photochemical optical bench (POB) is composed of a light source, a lens, glass or chemical solution filters, and a reaction vessel. The component parts are mounted on a rail in the optical bench. A typical POB apparatus is depicted in the following Figure 2:

FIGURE 2—A TYPICAL OPTICAL TRAIN



A TYPICAL OPTICAL TRAIN, WHERE E: POINT SOURCE ARC; L: LENS; F: SOLUTION AND GLASS FILTERS; $R_1$: REACTION CELL; $R_2$: ACTINOMETER CELL; $V_a$: THERMOSTAT VESSEL; W: QUARTZ WINDOW

A typical optical train, where E: point source arc;

L: lens; F: solution and glass filters; $R_1$: reaction cell;

$R_2$: actinometer cell; $V_a$: thermostat vessel; W: quartz window.

The light source is located at the end of the bench and contains a housing around a light source and a lens to collimate the beam. The light passes through glass filters and/or through cells containing filter solutions to transmit wavelength. Filter solutions in the cell may be circulated through a cooling system and the glass filters should be cooled with a stream of air to prevent heat buildup. The photochemical reaction vessel containing the test chemical solution or actinometer solution is mounted coaxially with the lamp on the bench so that the filtered light enters the window of the reaction vessel. The reaction vessel should be temperature controlled by circulating constant temperature water through side walls. The light flux is usually measured before and after the photolysis of the test chemical solution. Commercially available optical benches are highly recommended but simple "home-made" benches can be used [Andre (1977) under paragraph (d)(1) of this section]. If a "home-made" bench is used, it must be completely described.

(B) *Light sources.* There are a number of light sources which are available for use in photochemical studies: for example, low, medium, and high pressure mercury lamps; xenon lamps; and lasers. The characteristics and application of these lamps are described in detail by Calvert and Pitts (1966) under paragraph (d)(2) of this section, Murov (1973) under paragraph (d)(10) of this section, and de Mayo and Shizuka (1976) under paragraph (d)(3) of this section. The use of a POB or PMGRR will often narrow the choice of lamp with regard to use of a point source or a tube-type lamp, since the former can be focused to give a collimated beam while the latter cannot. The light source should emit light at a constant and high intensity. The 450 watt medium pressure mercury lamp is highly recommended for use in this test method.

(C) *Light filtering systems.* (*1*) Monochromatic or narrow band wavelength light is essential for the accurate determination of the reaction quantum yield of a test chemical. Various systems for isolating monochromatic or narrow band wavelengths are described by Calvert and Pitts (1966) under paragraph (d)(2) of this section, Murov (1973) under paragraph (d)(10) of this section, and de Mayo and Shizuka (1976) under paragraph (d)(3) of this section. Two filter systems commonly used to isolate the 313 and 366 bands from a 450 watt medium pressure mercury lamp are highly recommended and have been incorporated in this test method. These two filter systems are described as follows:

(*i*) 313 nm filtering system: Corning Glass CS-754 filter (if the test chemical does not absorb light at wavelengths greater than 400 nm, this filter is not needed. The 754 filter is designed to block out visible light) with 0.005 M potassium chromate solution containing 3 percent sodium carbonate.

(*ii*) 366 nm filtering system: Corning Glass CS 0-52 and CS 7-60 filters.

(*2*) Reagent-grade chemicals should be used to prepare the chemical filter solution. Since this filter solution (and in general any filter solution) degrades slowly over prolonged periods of photolysis, the solution should be carefully monitored. Even when tap water is used to cool the lamp, the buildup of solid material or algae may reduce the light intensity; and this must be checked repeatedly.

(D) *Reaction cells.* (*1*) In general, reaction cells of large volume are appropriate for POB equipment, while small reaction cells are used for PMGRR equipment. These cells should be constructed of borosilicate glass or quartz. Actinometer and test chemical solutions must be photolyzed in identical cells and should contain the same volumes of actinometer and test chemical solution.

(*2*) For the PMGRR equipment, disposable culture tubes (13 × 100 mm) with Teflon-lined screw caps or quartz tubes (13 × 100 mm) with quartz ground glass stoppers or borosilicate screw caps, Teflon-lined, are recommended for use as reaction tubes. Grease should be avoided since hydrophobic chemicals might adsorb to it. In carrying out the photolysis experiments, one tube is used for each datum point measured. The pathlength of these cells is discussed in paragraph (b)(1)(iii) of this section.

(*3*) For the POB equipment, the most common and functional design for reaction cells is a cylindrical shape with optically flat circular windows fused to each end of the cylinder and at right angles to its axis. The windows should be made of material that will transmit 100 percent of the light at the desired wavelengths. Optically flat quartz windows are recommended. The size of the cylindrical reaction cell and windows should be consistent with the dimensions of the light beam used in the equipment. The reaction cell should be of sufficient volume to permit removal of samples for analysis without significantly altering the volume of the reaction solution in the cell. The cell pathlength can be measured directly or it can be determined by the procedure discussed in paragraph (b)(1)(iii) of this section. Details for the construction of these reaction cells may be found in Calvert and Pitts (1966) under paragraph (d)(2) of this section and de Mayo and Shizuka (1976) under paragraph (d)(3) of this section.

(iii) *Cell pathlength.* Zepp (1978) under paragraph (d)(13) of this section, described an experimental method using an isolated wavelength band to determine the effective pathlength of any cell. This procedure is described in the public record for this section. This procedure has been used to measure the effective pathlength of Corning Glass culture tubes 13 × 100 mm and it was found that l was 11.2 mm [Mill et al. (1982) under paragraph (d)(9) of this section]. In a similar manner, reaction tubes made from borosilicate glass stock of O.D. 12 mm had an effective pathlength of 10.0 mm [Mill et al. (1982) under paragraph (d)(9) of this section]. This procedure can be used to measure the pathlength of rectangular cells designed for the POB apparatus. However, it is recommended that the pathlength of the rectangular cells be measured directly with a precise centimeter ruler or an equivalent measuring device.

(iv) *Solvents.* (A) If the half-life of an aqueous solution of test chemical in the photochemical equipment is less than 24 hours, then distilled water meeting ASTM Type II standards, or an equivalent grade, is recommended for use in this test method. If the half-life of an aqueous solution of test chemical in the photochemical equipment is greater than 24 hours, then water meeting ASTM type IIA standards, or an equivalent grade, is highly recommended for use in this test method to minimize biodegradation. ASTM Type II and IIA water are described in ASTM D 1193-77 (Reapproved 1983). Air saturated water is required for photolysis of test chemical solutions. Air saturated water can be easily prepared by allowing the ASTM Type II water to equilibrate in a vessel plugged with cotton or ASTM Type IIA water to equilibrate in a vessel plugged with sterile cotton. ASTM D 1193-77 (Reapproved 1983) is available for inspection at the Office of the Federal Register, Rm. 8401, 1100 L St., NW., Washington, DC. This incorporation by reference was approved by the Director of the Office of the Federal Register. This material is incorporated as it exists on the date of approval and a notice of any change in this material will be published in the FEDERAL REGISTER. Copies of the incorporated material may be obtained from the TSCA Public Docket Office (TS-793), Rm. NE-G004, Office of Toxic Substances, Environmental Protection Agency, 401 M St., SW., Washington, DC 20460, or from the American Society for Testing and Materials (ASTM), 1916 Race Street, Philadelphia, PA 19103.

(B) Reagent-grade acetonitrile is recommended as the organic cosolvent with water in photochemical studies. Spectrograde acetonitrile or methanol is recommended for spectroscopic studies to determine the molar absorptivity of the test chemical.

(v) *Sterilization.* If the half-life of an aqueous solution of test chemical in the photochemical equipment is greater than 24 hours, then it is important to sterilize all glassware and to use aseptic conditions in the preparation of all solutions for photolysis studies in order to eliminate or minimize biodegradation. Glassware can be sterilized in an autoclave or by any other suitable non-chemical method.

(vi) *pH effects.* It is recommended that all photolysis and molar absorptivity experiments be carried out at pHs at least two pH units above the $pK_a$ and at least two pH units below the $pK_a$ for any chemical that ionizes or protonates (e.g., carboxylic acids, phenols, and amines). Buffers described in paragraph (b)(2)(i) of this section should be used.

(vii) *Volatile chemical substances.* Special care should be taken when testing a volatile chemical so that the chemical substance is not lost due to volatilization during the course of the photolysis experiment. Thus, it is important to effectively seal the reaction vessels. Disposable culture tubes with Teflon-lined screw caps or quartz tubes with quartz or borosilicate screw caps, Teflon-lined, are recommended. Grease should not be used. Volatile compounds can be conveniently studied in culture tubes equipped with gas-tight Mininert* valves. Samples can be introduced into or removed from the tubes through the septum in these valves with no loss of substrate. As an alternative, the tubes can be sealed with a torch. In addition, the reaction vessels should be as completely filled as is possible to minimize volatilization to any air space.

(viii) *Control solution.* It is extremely important to take certain precautions to prevent loss of chemical from the reaction vessels by processes other than photolysis. For example, biodegradation and volatilization can be eliminated or minimized by use of sterile conditions and minimal air space in sealed vessels. Hydrolysis is a process which cannot be minimized by such techniques. Thus, control vessels containing test substance which are not exposed to light are required. In this way, the loss of test chemical in processes other than photolysis may be determined and eliminated or minimized. For simplicity, if the loss of chemical in the control is small (i.e., approximately 10 percent or less), one can calculate a first-order loss, $k_{loss}$, and subtract it from $(k_p)_{obs}$ to give the corrected direct photolysis rate constant $K_p$. If hydrolysis is found to be significant (greater than 10 percent), hydrolysis studies should be carried out first under § 796.3500.

(ix) *Absorption spectrum as a criterion for performing the reaction quantum yield experiments.* This aqueous photolysis test is applicable to all chemicals which have UV-visible absorption maxima in the range 290–800 nm. Some chemicals have absorption maxima significantly below 290 nm but have measurable absorption tails above the baseline in their absorption spectrum at wavelengths greater than 290 nm. Photolysis experiments should be carried out for these chemicals to determine the reaction quantum yield. The absorption spectrum of the chemical in aqueous solution can be measured by the procedures given in § 796.1050.

(x) *Actinometers.* Chemical actinometers are used in reaction quantum yield experiments to measure the integrated light intensity incident on the sample during photolysis. Chemical actinometers have photochemical reactions which have well-defined reaction quantum yields $\phi_\lambda$ at wavelength λ.

(A) *Low optical density actinometers.* Two low optical density actinometers (absorbance <0.02) are described which can be used to determine the reaction quantum yield of a test chemical at low optical density (absorbance < 0.02) in Procedure One, under paragraph (b)(2) of this section. These actinometers are: p-nitroacetophenone-pyridine actinometer (PNAP/PYR); and p-nitroanisole-pyridine actinometer (PNA/PYR). The rate constants and half-lives of these actinometers can be adjusted to match the rate constant and half-life of the test chemical by adjusting the concentration of pyridine. With a 450-watt medium pressure mercury lamp in an Ace-type PMGRR, the PNAP/PYR actinometer can be adjusted with pyridine to have half-lives that range from greater than 12 hours to several weeks while the PNA/PYR actinometer can be adjusted with pyridine to have half-lives that range from approximately 15 minutes to approximately 12 hours (if a Moses-type PMGRR is used, then new criteria have to be defined to determine which of these two actinometers should be used. Details are in the public record for this section).

(*1*) *p-Nitroacetophenone-pyridine actinometer (PNAP/PYR).* (*i*) The reaction quantum yield of the PNAP/PYR actinometer is a function of the molar concentration of pyridine [PYR] and this relationship is given by Equation 7 under paragraph (a)(3)(ii)(A)(*3*) of this section. This equation is valid up to 0.2 M pyridine. The initial concentration of PNAP $(C_o)$ is precisely set in the range of approximately 1 x $10^{-5}$ M.

The molar absorptivities of PNAP at 313 and 366 nm are: $\epsilon_{313_e}$ = 2,056 $M^{-1}$ $cm^{-1}$; and $\epsilon_{366_e}$ = 160 $M^{-1}$ $cm^{-1}$. The chemical analysis of PNAP is given in paragraph (b)(1)(xi)(B) of this section.

(*ii*) Trial photolysis experiments are required to determine the concentration of pyridine needed to adjust the rate constant of the actinometer to approximately equal the rate constant of the test chemical (within ± 50 percent). In the Phase 2 experiments of Procedure 1 under paragraph (b)(2)(ii) of this section, the rate constant of the test chemical $k_{pc}$ is determined in the Ace-type PMGRR with a 450-watt medium pressure mercury lamp. As an approximation, for the PNAP/PYR actinometer in the PMGRR with a 450 watt medium pressure mercury lamp, the concentration of pyridine needed to adjust the rate constant of the actinometer to approximately equal the rate constant of the test chemical at 313 and 366 nm is given by the following equations:

*Equation 18*

at 313 nm $[PYR]=1.16\ k_{pc}$

*Equation 19*

at 366 nm $[PYR]=5.95k_{pc}$.

where $k_{pc}$ is in the units of $(hours)^{-1}$ and [PYR] is the molar concentration of pyridine.

(*iii*) A trial experiment is then carried out in the Phase 2 procedure under paragraph (b)(2)(ii) of this section by preparing the actinometer with the molar concentration of pyridine [PYR] estimated from Equations 18 or 19 under paragraph (b)(1)(X)(A)(*1*)(*ii*) of this section and photolyzing the actinometer solution at a fixed molar concentration of PNAP ($C_o$) in the PMGRR at 313 or 366 nm. After approximately 50 percent transformation, the concentration of PNAP ($C_t$) is recorded at time t and an approximate rate constant $k_{pe}$ is calculated using these data in Equation 2 under paragraph (a)(3)(ii)(A)(*1*) of this section. This trial experiment is repeated by adjusting the molar concentration of pyridine [PYR] until the rate constant of the test chemical and actinometer are approximately equal.

(*2*) *p-Nitroanisole-Pyridine Actinometer (PNA/PYR).* (*i*) The reaction quantum yield of the PNA/PYR actinometer is a function of the molar concentration of pyridine [PYR] and this relationship is given by Equation 8 under paragraph (a)(3)(ii)(A)(*3*) of this section. This equation is valid up to 0.02 M pyridine. The initial concentration of PNA ($C_o$) is precisely set in the range of approximately 1 x $10^{-5}$ M at 366 nm and at approximately 0.4 x $10^{-5}$ M at 313 nm. The molar absorptivities of PNA at 313 and 366 nm are: $\epsilon_{313_e}$ = 10,300 $M^{-1}$ $cm^{-1}$; and $\epsilon_{366_e}$ = 1,990 $M^{-1}$ $cm^{-1}$. The chemical analysis for PNA is given in paragraph (b)(1)(xi)(C) of this section.

(*ii*) Trial photolysis experiments are required to determine the concentration of pyridine needed to adjust the rate constant of the actinometer to approximately equal the rate constant of the test chemical (within ± 50 percent). In the Phase 2 experiments of Procedure 1 under paragraph (b)(2)(ii) of this section, the rate constant of the test chemical $k_{pc}$ is determined in an Ace-type PMGRR with a 450-watt medium pressure mercury lamp. As an approximation, for the PNA/PYR actinometer in the PMGRR with a 450 watt medium pressure mercury lamp, the concentration of pyridine needed to adjust the rate constant of the actinometer to equal the rate constant of the test chemical at 313 and 366 nm is given by the following equations:

*Equation 20*

at 313 nm $[PYR]=8.93 \times 10^{-3}\ (k_{pc}-0.0722)$

*Equation 21*

at 366 nm $[PYR]=1.85 \times 10^{-3}\ (k_{pc}-0.0349)$

where $k_{pc}$ is in the units of $(hours)^{-1}$ and [PYR] is the molar concentration of pyridine.

(*iii*) A trial experiment is then carried out in the Phase 2 procedure under paragraph (b)(2)(ii) of this section by preparing the actinometer with the molar concentration of pyridine [PYR] estimated from Equation 20 or 21 under paragraph (b)(1)(X)(A)(*2*)(*ii*) of this section and photolyzing the actinometer solution at a fixed molar concentration of PNA ($C_o$) in the Ace-type PMGRR at 313 or 366 nm. After approximately 50 percent transformation, the concentration of PNA ($C_t$) is recorded at time t and an approximate rate constant $k_{pe}$ is calculated using these data in Equation 2 under paragraph (a)(3)(ii)(A)(*1*) of this section. This trial experiment is repeated by adjusting the molar concentration of pyridine [PYR] until the rate constant of the test chemical and actinometer are approximately equal.

(B) *High optical density actinometer (ferrioxalate actinometer).* The ferrioxalate actinometer at high optical density (absorbance >2) is a widely used actinometer by photochemists and has been recommended for use in Procedure Two under paragraph (b)(3) of this section [Murov (1973) under paragraph (d)(10) of this section, de Mayo and Shizuka (1976) under paragraph (d)(3) of this section, Calvert and Pitts (1966) under paragraph (d)(1) of this section]. This actinometer is applicable over the environmentally relevant range 290 to 500 nm. The net photochemical reaction is

*Equation 22*

$$Fe^{3+} + (C_2O_4^{2-})/2 \; h\gamma \; Fe^{2+} + CO_2.$$

At high optical density, the reaction kinetics are zero-order and the kinetics are followed by measuring the molar concentration of $Fe^{2+}$ formed as a function of time t using Equation 11 under paragraph (a)(3)(ii)(B)(1) of this section. The $Fe^{2+}$ formed is measured by procedures outlined in paragraph (b)(1)(xi)(D) of this section. Murov (1973) under paragraph (d)(10) of this section gives a detailed procedure for using this actinometer and it is highly recommended. This procedure should be modified slightly to use the ferrioxalate at 0.15 M. The irradiated solution is diluted 100-fold prior to analysis for ferrous ion, which must not be allowed to exceed 0.005 M. Table 1 of the associated Technical Support Document, which is part of the public record for this section, lists the reaction quantum yield as a function of wavelength. At 0.15 M, the quantum yield ($\phi_\lambda$) at 313 and 366 nm is 1.20 and 1.18, respectively. All ferrioxalate actinometry experiments must be carried out in a darkroom with photographic "safelights." Ferrioxylate is available from Alfa Inorganics. If the test chemical absorbs light at wavelengths greater than 500 nm, then Reinicke's salt can be used as the actinometer. The use of Reinicke's salt actinometer is described in the public record for this section.

(xi) *Chemical analysis of solutions*—(A) *Chemical analysis of test chemical solutions.* In determining the concentration of the chemical in solution, an analytical method should be selected which is most applicable to the analysis of the specific chemical substance. Chromatographic methods are generally recommended because of their chemical specificity in analyzing the parent chemical substance without interference from impurities. Whenever practicable the chosen analytical method should have a precision of ± 5 percent or better.

(B) *Chemical analysis of p-nitroacetophenone (PNAP).* The p-nitroacetophenone (PNAP) in the chemical actinometer solution is conveniently analyzed by high-pressure liquid chromatography using a 30 cm $C_{18}$ reverse-phase column and a UV detector set at 280 nm. The mobile phase in volume percent is 2.5 percent acetic acid, 50 percent acetonitrile, and 47.5 percent water which is passed through the column at a flow rate of 2 mL/minute. [Mill et al. (1982) under paragraph (d)(8) of this section and Mill and Dulin (1982) under paragraph (d)(4) of this section.

(C) *Chemical analysis of p-nitroanisole (PNA).* The p-nitroanisole (PNA) in the chemical actinometer solution is conveniently analyzed by high-pressure liquid chromatography using a 30 cm $C_{18}$ reverse-phase column and a uv detector set at 280 nm. The mobile phase in volume percent is 50 percent acetonitrile and 50 percent water which is passed through the column at a flow rate of 2 mL/minute [Dulin and Mill (1982) under paragraph (d)(4) of this section].

(D) *Chemical analysis of Ferrous Ion in the Ferrioxalate Actinometer.* The concentration of $Fe^{2+}$ formed in the photolysis of the ferrioxalate actinometer is measured spectrophotometrically via the formation of a red phenanthroline complex and determining the absorbance of the complex at 510 nm. Murov (1973) under paragraph (d)(10) of this section describes a detailed procedure for measuring the molar concentration of ferrous ion formed in the photolysis reaction and this procedure is highly recommended. This procedure has been modified slightly to use the ferrioxalate at 0.15 M. As a result, the irradiated solution must be diluted 100-fold prior to analysis for ferrous ion, which must not be allowed to exceed 0.005 M.

(2) *Procedure one: Determination of the reaction quantum yield by the low optical density test chemical and actinometer method*—(i) *Phase 1 Experiments: UV-Visible Absorption Spectra.* The UV-visible absorption spectra in aqueous solution can be determined by the methods described in §§ 796.1050 and 796.3700. It is recommended that the following additional procedures be followed:

(A) For chemicals which ionize or protonate (e.g., carboxylic acids, phenols, amines), carry out UV-visible absorption studies at pHs at least two pH units above the $pK_a$ and at least two pH units below the $pK_a$. Prepare buffer solutions at 25 °C using reagent grade chemicals and distilled water as follows:

pHs in the range 3–6[1]: $NaH_2PO_4$, HCl;
pHs in the range 6–8[1]: $KH_2PO_4$, NaOH;
pHs in the range >8: Prepare buffers as described in the Handbook of Chemistry and Physics.

Check the pH of all the buffer solutions with a pH meter at 25 °C and adjust to the proper pH, if necessary. These buffer solutions can then be added to the test chemical solution until the desired pH is obtained. If these buffers are inadequate, then adjust the pH of the test chemical solution with 0.01 M HCl or NaOH at 25 °C.

(B)(*1*) Measure the absorbance, $A_{\lambda_c}$, as a function of wavelength in the range 290 to 800 nm in duplicate. If applicable, measure $A_{\lambda_c}$ at each experimental pH under paragraph (b)(1)(vi) of this section. Record, in duplicate, the baseline where both the sample and reference cells are filled with blank solutions. These data will be used to calculate the molar absorptivities for the appropriate intervals and wavelength centers, under § 796.3700(c)(3) Table 1, where the test chemical absorbs light. The wavelength center is defined as the midpoint of the interval range listed in §796.3700 Table 1.

(*2*) Measure $A_{\lambda_c}$ at 313.0 nm and 366.0 nm in duplicate. These data will be used to calculate the molar absorptivities of the test chemical, $\epsilon_{313_c}$ and $\epsilon_{366_c}$. Photolysis experiments should be carried out at the wavelength corresponding to the higher value of the molar absorptivity.

(*3*) It must be emphasized that the molar absorptivities of the test chemical ($\epsilon_{\lambda_c}$) must be carefully determined, especially in the tails of the absorption bands at $\lambda > 290$ nm. Large errors will occur when calculating photolysis rate constants and half-lives if these measurements are not carefully carried out.

(ii) *Phase 2 experiments: Trial photolysis experiments*—(A)—*Determination of the approximate rate constant of the test chemical*—(*1*) *Preparation of buffer solutions.* Prepare buffer solutions according to the procedures outlined in paragraph (b)(2)(i) of this section using reagent grade chemicals and pure water as described under Test Conditions in paragraph (b)(1)(iv) of this section, for chemicals that reversibly ionize or protonate.

(*2*) *Preparation of Test Chemical Solution.* Prepare a homogeneous solution of test chemical below its water solubility and at an absorbance less than 0.02 in the photolysis reaction vessel at 313 or 366 nm. For very hydrophobic chemicals, it is difficult and time consuming to prepare aqueous solutions. To facilitate the preparation of aqueous solutions containing very hydrophobic chemicals and to allow for easier analytical procedures, the following procedure may be used as an aid in the dissolution of the test chemical. Dissolve the pure test chemical in reagent grade acetonitrile. Add pure water as described under Test Conditions in paragraph (b)(1)(iv) of this section, or buffer solution, as described in paragraph (b)(2)(iv) of this section, for chemicals which ionize or protonate, to an aliquot of the acetonitrile solution. Do not exceed one-volume percent of acetonitrile in the final solution.

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[1] Use the minimum concentration of buffers to attain the desired pH.

(*3*) *Performance of the test.* (*i*) Prepare an aqueous solution of test chemical as described in paragraph (b)(2)(ii)(A)(*2*) of this section and a sufficient number of samples in quartz or borosilicate glass tubes under paragraph (b)(1)(ii)(D) of this section to perform all the required tests. Measure the initial concentration of test chemical ($C_o$) as described in paragraph (b)(1)(xi)(A) of this section in duplicate. Fill the tubes as completely as possible and seal them. Do not use grease. Prepare two control samples in the absence of ultraviolet light and totally exclude light by wrapping the tubes with aluminum foil or by any other suitable method in paragraph (b)(1)(viii) of this section. Place the samples, including the controls, in the Ace-type PMGRR with a 450 watt medium pressure mercury lamp and appropriate filters as described in paragraphs (b)(1)(ii)(A) and (B) of this section. The reaction tubes should be controlled to a temperature of $t_1$ ± 2° C within the range 20 to 30° C. Photolyze the samples at 313 or 366 nm corresponding to the higher value of the molar absorptivity of the test chemical ($\epsilon_{313c}$ or $\epsilon_{366c}$ determined in the Phase 1 experiments, under paragraph (b)(2)(i) of this section) until approximately 50 percent of the test chemical has transformed. Measure the molar concentration of the test chemical ($C_t$) in duplicate at time t. For test chemicals that ionize or protonate, carry out the photolysis experiments at the required pHs as described under Test Conditions in paragraph (b)(1)(vi) of this section.

(*ii*) After the photolysis experiments are completed, determine the concentration of test chemical in the controls in duplicate. If a significant loss of test chemical has occurred in the controls, determine the cause and eliminate, or minimize, the loss. If hydrolysis is found to be significant, hydrolysis experiments should be carried out first under paragraph (b)(1)(viii) of this section.

(*iii*) The data obtained in this section will be used to determine an approximate $k_{pc}$ and $(t_{1/2})_c$ using Equations 3 and 5 under paragraph (a)(3)(ii)(A)(*1*) of this section.

(B) *Actinometry Experiments.* These experiments are designed to choose the appropriate low optical density actinometer [i.e., PNAP/PYR under paragraph (b)(1)(x)(A)(*1*) of this section or PNA/PYR under paragraph (b)(1)(x)(A)(*2*) of this section] and to adjust the rate of the chosen actinometer so that the rate constant of the actinometer ($k_{pa}$) is approximately equal to the rate constant of the test chemical ($k_{pc}$). Based on the photolysis experiments in paragraph (b)(2)(ii) of this section, if $(t_{1/2})_c$ is less than 12 hours, use the PNA/PYR actinometer; if $(t_{1/2})_c$ is greater than 12 hours, use the PNAP/PYR actinometer.

(*1*) *Preparation of actinometer solutions*—(*i*) *PNAP/PYR Actinometer.* Using the test chemical photolysis rate constant $k_{pc}$ determined in paragraph (b)(2)(ii)(A) of this section and equations 18 or 19 under paragraph (b)(1)(x)(A)(*1*)(*ii*) of this section, determine the molar concentration of pyridine needed to adjust the rate constant of the actinometer to approximately equal the rate constant of the test chemical at the appropriate wavelength 313 or 366 nm (chosen based on the results of the Phase 1 experiments). Once the molar concentration of pyridine [PYR] has been estimated, the actinometer solution can be prepared as follows. Dissolve 0.0165 grams of PNAP in 10 mL of acetonitrile (0.01 M). Add 1 mL of this solution to a 1 liter volumetric flask. Add to the volumetric flask the mass in grams, or the volume V of pyridine at 20 °C, obtained from the equations

*Equation 23*

$$\text{mass (grams)} = 79.1\,[\text{PYR}]$$

*Equation 24*

$$V(\text{mL}) = 80.6[\text{PYR}]$$

Fill the volumetric flask with pure water as described in paragraph (b)(1)(iv) of this section to give 1 liter of solution and shake vigorously to make sure that the solution is homogeneous. The resultant concentration of PNAP is $1.00 \times 10^{-5}$ M. The PNAP/PYR solution should be wrapped with aluminum foil and kept from bright light.

(*ii*) *PNA/PYR actinometer.* Using the test chemical photolysis rate constant $k_{pc}$ determined in paragraph (b)(2)(ii)(A) of this section and Equations 20 or 21 under paragraph (b)(1)(4)(A)(2) of this section, determine the concentration of pyridine needed to adjust the rate constant of the actinometer to approximately equal the rate constant of the test chemical at the appropriate wavelength 313 or 366 nm (chosen based on the results of the Phase 1 experiments). Once the molar concentration of pyridine has been determined, an actinometer solution can be prepared as follows. If photolysis experiments are to be carried out at 366 nm, dissolve 0.0153 grams of PNA in 10 mL of acetonitrile (0.01 M). Add 1 mL of this solution to a 1 liter flask. Add to the volumetric flask the mass in grams, or the volume of pyridine at 20 ° C as defined by equations 23 and 24 under paragraph (b)(2)(ii)(B)(*1*)(*i*) of this section. Fill the volumetric flask with pure water under paragraph (b)(1)(iv) of this section to give 1 liter of solution and shake vigorously to make sure that the solution is homogeneous. The resultant concentration is 1.00 x $10^{-5}$ M. The PNA/PYR solution should be wrapped with aluminum foil and kept from bright light. If photolysis experiments are to be carried out at 313 nm, dissolve 0.00612 grams of PNA in 10 mL of acetonitrile (0.004 M). Then follow the procedure described above. The resultant PNA concentration is 0.400 x $10^{-5}$ M. The PNA/PYR solution should be wrapped with aluminum foil and kept from bright light.

(*2*) *Performance of the test.* Prepare a solution of the appropriate actinometer as described in paragraph (b)(2)(ii)(B)(*1*) of this section. Measure the initial molar concentration of actinometer $C_o$ [PNAP or PNA as described in paragraphs (b)(1)(xi) (B) and (C) of this section]. Prepare a sufficient number of samples in borosilicate or quarts tubes, under paragraph (b)(1)(ii)(D) of this section, to perform all the required tests. The tubes should be identical to the tubes used to determine $k_{pc}$ in paragraph (b)(2)(ii)(A)(*3*) of this section. Fill the tubes as completely as possible and seal them. It is important that the actinometer tubes contain the same volume as that used in the test chemical tubes, paragraph (b)(2)(ii)(A)(*3*) of this section. Prepare two control samples in the absence of ultraviolet light and totally exclude light by wrapping the tubes with aluminum foil or by any other suitable method under paragraph (b)(1)(viii) of this section. Place the samples, including the controls, in the Ace-type PMGRR with the 450 watt medium pressure mercury lamp and the appropriate filters. The tubes should be controlled to a temperature $t_1 \pm 2$ °C. Photolyze the samples at the chosen wavelength λ (313 or 366 nm) until approximately 50 percent of the actinometer has transformed. Determine the concentration of the actinometer ($C_t$) in duplicate by the appropriate procedure described in paragraphs (b)(1)(xi) (B) and (C) of this section at time t. Calculate a rate constant using Equation 2 under paragraph (a)(3)(ii)(A)(*1*) of this section. If the rate constant $k_{pa}$ is not approximately the same as the rate constant of the test chemical $k_{pc}$, then repeat the experiment with an appropriate concentration of pyridine until $k_{pa}$ is approximately equal to $k_{pc}$ (within ± 50 percent).

(iii) *Phase 3 experiments: Determination of the reaction quantum yield of the test chemical.* Based on the results of the Phase 1 and 2 experiments, use the appropriate actinometer with the required concentration of pyridine to make the rate constant of the actinometer approximately match the rate constant of test chemical. Follow the procedure outlined in paragraph (b)(2)(ii)(B)(*1*) of this section to prepare the actinometer and a sufficient number of samples in borosilicate glass or quartz tubes as described under paragraph (b)(1)(ii)(D) of this section to perform all the required tests. Determine the initial concentration of the actinometer ($C_o$), in duplicate according to the appropriate procedure in paragraph (b)(1)(xi) of this section. Fill all the tubes as completely as possible, seal them, and cover half of the tubes with aluminum foil as soon as possible after preparation.

Prepare an aqueous solution of test chemical as described in paragraph (b)(2)(ii)(A)(*2*) of this section and determine the initial concentration of test chemical ($C_0$), in duplicate according to the procedure described in paragraph (b)(1)(xi)(A) of this section. Prepare a sufficient number of samples in borosilicate or quartz tubes to perform all the required tests. Fill all the tubes as completely as possible, seal them, and cover half of the tubes with aluminum foil as soon as possible after preparation. The reaction tubes for actinometer and test chemical must be identical and contain the same volume of solution. Place the tubes to be photolyzed in the Ace-type PMGRR containing a 450 watt medium pressure mercury lamp and the appropriate filters, paragraphs (b)(1)(ii) (B) and (C) of this section, and all the control tubes close to the PMGRR. The tubes should be temperature controlled to a temperature $t_1$ ± 2 °C. Based on the results of the Phase 2 experiments, determine the concentration of test chemical and actinometer periodically between 10 and 80 percent transformation in duplicate [at least 6 data points at approximately equal times]. Determine the concentration of actinometer and test chemical in the controls in duplicate at each time point.

(3) *Procedure Two: Determination of the reaction quantum yield by the low optical density test chemical and high optical density actinometer method*—(i) *Phase 1 Experiments: UV-Visible Absorption Spectra.* Follow the procedure outlined in paragraph (b)(2)(i) of this section and measure the absorbance of the test chemical, $A_{\lambda_c}$, as a function of wavelength in the range 290 to 800 nm in duplicate. Measure $A_{\lambda_c}$ at 313.0 and 366.0 nm in duplicate. The absorbance data at 313.0 and 366.0 nm will be used to calculate the molar absorptivities of the test chemical, $\epsilon_{313_c}$ and $\epsilon_{366_c}$, and photolysis experiments should be carried out at the wavelength corresponding to the highest value of the molar absorptivity.

(ii) *Phase 2 experiments: Determination of the reaction quantum yield of the test chemical*—(A) *Preparation of buffer solutions.* Prepare buffer solutions according to the procedures outlined in paragraph (b)(2)(ii)(A)(*1*) of this section for a test chemical which ionizes or protonates.

(B) *Preparation of test chemical solution.* Prepare a homogeneous solution of test chemical according to the procedures outlined in paragraph (b)(2)(ii)(A)(*2*) of this section.

(C) *Preparation of ferrioxalate actinometer solution.* Prepare a ferrioxalate actinometer solution at 0.15 M as described in paragraph (b)(1)(x)(B) of this section following the procedure outlined by Murov (1973) under paragraph (d)(10) of this section which has been modified slightly. Since the ferrioxalate actinometer solution is very sensitive to visible light, all these actinometer experiments must be carried out in a darkroom with photographic "safelights."

(D) *Determination of the quantum yield of the test chemical.* (*1*) Prepare a solution of the ferrioxalate actinometer at high optical density (absorbance >2) as described in paragraph (b)(3)(ii)(C) of this section and completely fill the reaction cell described in paragraph (b)(1)(ii)(D) of this section. Place the reaction cell in the photochemical optical bench (POB) as described in paragraph (b)(1)(ii)(A)(*2*) of this section with a 450 watt medium pressure mercury lamp under paragraph (b)(1)(ii)(B) of this section and appropriate filters under paragraph (b)(1)(ii)(C) of this section. The reaction cell should be controlled to a temperature of $t_1$±2 °C in the range 20 to 30 °C. The filter system used should isolate the wavelength 313 or 366 nm corresponding to the larger value of $\epsilon_{313_c}$ or $\epsilon_{366_c}$ determined in the Phase 1 experiments. Photolyze the actinometer solution in the reaction cell for 1, 3, 6, 9, 12, and 15 minutes and measure the concentration of $Fe^{2+}$ formed spectrophotometrically by the procedure outlined in paragraph (b)(1)(xi)(D) of this section in duplicate at each of the time points. No time points should be taken when the optical density of the actinometer falls below 2. Only withdraw small volumes for analysis so that the total volume of the actinometer solution in the reaction cell does not change appreciably. This procedure is repeated in a third set of experiments soon after the test chemical photolysis experiments are performed.

(*2*) In the second set of experiments, a solution of test chemical is prepared according to the procedure outlined in paragraph (b)(3)(ii)(B) of this section at low optical density (absorbance <0.02). Measure the initial concentration $(C_o)_c$ in duplicate by the procedure outlined in paragraph (b)(1)(xi)(A) of this section. Fill two reaction cells with the same volume as that used in the actinometer experiments. One of these cells is wrapped with aluminum foil, placed close to the POB and temperature controlled to $t_s$±2 °C. The second reaction cell (which is the same one used for the actinometry experiments) is placed in the POB and photolyzed at $t_s$± 2 °C at the same wavelength used in the actinometry experiments. Determine the concentration of test chemical periodically between 10 and 80 percent transformation in duplicate [at least 6 data points at approximately equal times]. Only withdraw small volumes for analysis so that the total volume of the test chemical solution in the reaction cell does not change appreciably. Determine the concentration of the control in duplicate at each time point.

(*3*) As an alternative procedure, the same series of experiments described above can be carried out in a PMGRR using individual reaction tubes for each datum point.

(c) *Data and reporting*—(1) *Procedure One: Determination of the reaction quantum yield by the low optical density test chemical and actinometer method*—(i) *Phase 1 Experiments: UV-Visible Absorption Spectra*—(A) *Treatment of results.* (*1*) The molar absorptivity ($\epsilon_{\lambda c}$) can be determined from the absorption spectra using the expression

*Equation 25*

$$\epsilon_{\lambda c}=A_{\lambda c}/Cl,$$

where $A_{\lambda c}$ is the absorbance at wavelength λ, C is the molar concentration of test chemical, l is the cell pathlength in centimeters. The molar absorptivity of the chemical should be determined for the wavelengths listed in § 796.3700(c)(3) Table 1, for a solution of concentration C and in a cell with pathlength l. If the absorption curve is flat within the interval around the wavelength center, $\epsilon_{\lambda c}$ may be determined from the absorbance $A_{\lambda c}$ at λ center using Equation 25. If a large change in absorbance occurs within this interval, obtain an average $A_{\lambda c}$ at λ center based on the two absorbances at the boundary of the interval. Calculate an average $\epsilon_{\lambda c}$ using the average value of $A_{\lambda c}$ in Equation 25. Determine the molar absorptivity for each replicate and calculate a mean value.

(*2*) Determine the molar absorptivity of the test chemical at 313.0 and 366.0 nm for a solution of test chemical at a molar concentration C and in a cell of pathlength l using Equation 25 under paragraph (c)(1)(i)(A)(*1*) of this section. Determine $A_{\lambda c}$ and the molar absorptivity at 313.0 and 366.0 nm for each replicate and calculate a mean value.

(*3*) Using the molar absorptivities obtained from the spectra and the values of $L_\lambda$ from § 796.3700(c)(3) Tables 3 to 6, calculate the maximum direct photolysis rate constant $(k_{pE})_{max}$ at a specific latitude (corresponding to the manufacturing site) and season of the year using Equation 16 under paragraph (a)(3)(ii)(B)(*6*) of this section. The corresponding minimum half-life, $(t_{1/2E})_{min}$ can then be calculated using this $(k_{pE})_{max}$ in Equation 17 under paragraph (a)(3)(ii)(B)(*6*) of this section.

(B) *Test data report.* (*1*) Report the name, structure, and purity of the test chemical.

(*2*) Submit a recent test spectrum on appropriate reference chemicals for photometric and wavelength accuracy.

(*3*) Submit the original chart, or photocopy, containing a plot of absorbance of test chemical vs. wavelength plus the baseline. Spectra should include a readable wavelength scale, preferably marked at 10 nm intervals. Each spectrum should be clearly marked with the test conditions.

(*4*) Report the concentration of the test chemical solution, the type of absorption cell used (quartz or borosilicate glass) and the pathlength.

(*5*) Report $A_{\lambda c}$ and $\epsilon_{\lambda c}$ at λ center for each replicate and the mean value.

(*6*) Report the identity and composition of the solvent used in the spectral absorption study.

(*7*) Report $\epsilon_{313c}$ and $\epsilon_{366c}$ along with $A_{313c}$ and $A_{366c}$ for each replicate and the mean value.

(*8*) Report $(k_{pE})_{max.}$ and $(tT2\frac{1}{2}_E)_{min.}$ for the summer and winter solstices using the appropriate $L_\lambda$ values from § 796.3700(c)(3) Tables 3–6, closest to the latitude of the chemical manufacturing site.

(*9*) For chemicals that ionize or protonate, report the data for steps 1 to 8 at the required pHs under paragraph (b)(1)(vi) of this section.

(*10*) For a chemical that ionizes or protonates, report its $pK_a$. Report the type and concentration of the buffers employed for each pH.

(*11*) Describe the method employed in determining the test chemical's concentration.

(*12*) Report the name and model of the spectrophotometer used.

(*13*) Report the various control settings employed with the spectrophotometer. These might include scan speed, slit width, gain, etc.

(*14*) If a Moses-type PMGRR was used in these experiments, describe it completely and report the aperture in cm$^2$.

(ii) *Phase 2 experiments: trial photolysis experiments*—(A) *Determination of the approximate rate constant of the test chemical*—(*1*) *Treatment of results.* From the photolysis experiments carried out at 313 or 366 nm (photolysis wavelength chosen based on the results of the Phase 1 experiments), use the concentration $(C_t)_c$ corresponding to approximately 50 percent of the initial molar concentration of chemical remaining at time t along with the initial molar concentration $(C_o)_c$ and the time t in hours in Equation 3 under paragraph (a)(3)(ii)(A)(*1*) of this section to calculate $k_{pc}$ in hours$^{-1}$. From the analysis of the two samples at time t=0 and t, calculate a mean value of $(C_o)_c$ and $(C_t)_c$, and a value of $k_{pc}$. If a slight loss of chemical has been detected in the controls, then calculate a rate constant as follows. Calculate an average concentration $(C_t)_c$ based on duplicate measurements of concentration in the controls at the end of the experiment (time t). Use this concentration along with the average initial concentration $(C_o)_c$ and t in Equation 3 under paragraph (a)(3)(ii)(A)(*1*) of this section and calculate a rate constant $k_{loss}$. Using this rate constant along with the observed rate constant in the photolysis experiments, the corrected rate constant is then

*Equation 26*

$$k_{pc}=(k_{pc})_{obs.}-k_{loss}$$

Calculate the half-life, $(t_{1/2})_c$ using the corrected value of $k_{pc}$ in Equation 5 under paragraph (a)(3)(ii)(A)(*l*) of this section.

(*2*) *Specific analytical and recovery procedures.* (*i*) Provide a detailed description or reference for the analytical procedures used, including the calibration data and precision; and

(*ii*) If extraction methods were used to separate the solute from the aqueous solution, provide a description of the extraction method as well as the recovery data.

(*3*) *Other test conditions.* (*i*) Report the size, approximate cell wall thickness, pathlength 1 , and type of glass used for the test chemical reaction tubes. If the cell pathlength was measured by the procedure described in the Technical Support Document available as part of the public record, report all the data obtained in these experiments.

(*ii*) Report the initial pH of all test solutions, if appropriate.

(*iii*) If acetonitrile was used to solubilize the test substance, report the percent, by volume.

(*iv*) If a significant loss of test chemical occurred in the control solution, indicate the causes and how they were eliminated or minimized.

(*4*) *Test data report.* (*i*) Report the wavelength used to photolyze the test chemical.

(*ii*) Report the initial molar concentration of test chemical $(C_o)_c$ of each replicate and the mean value.

(*iii*) Report the molar concentration of test chemical $(C_t)_c$ for each replicate and the mean value. Report the time t.

(*iv*) Report the molar concentration of each replicate control sample and the mean value after completion of the experiment. Report the time t.

(*v*) Report the value of $k_{pc}$ and $(t_{1/2})_c$. If small losses of chemical were observed, report $(k_{pc})_{obs.}$, $K_{loss}$, and $k_{pc}$. Report the half-life $(t_{1/2})_c$ calculated using the value of $k_{pc}$.

(*vi*) For chemicals that ionize or protonate, report the data for steps 1 through 5 for the experiments at the required pHs. Report the initial pH of all test chemical solutions and the type of concentration of the buffers used for each pH.

(B) *Actinometry experiments*—(*1*) Treatment of results. Follow the same discussion of the treatment of results, paragraph (c)(1)(ii)(A)(*1*) of this section, to determine the rate constant of the actinometer $k_{pa}$ using Equation 2 under paragraph (a)(3)(ii)(A)(*1*) of this section. Repeat these calculations for the trial actinometry experiments to determine the molar concentration of pyridine [PYR] needed to adjust the rate constant of the actinometer $(k_{pa})$ to be approximately equal to the rate constant of the test chemical $(k_{pc})$.

(*2*) *Test data report.* (*i*) Report the wavelength used to photolyze the actinometer.

(*ii*) Report the size, approximate cell wall thickness, pathlength l, and the type of glass used for the actinometer reaction tubes.

(*iii*) For each trial experiment: report the actinometer used; report the initial concentration of actinometer $(C_o)_a$ for each replicate and the mean value; report the molar concentration of the actinometer $(C_t)_a$ for each replicate and the mean value. Report the time t; report the molar concentration of each replicate control sample and the mean value after the completion of the experiment. Report the time t; and report the value of $k_{pa}$. If small losses were observed for the actinometer, report $(k_{pa})_{obs.}$, $k_{loss}$, and $k_{pa}$.

(*iv*) If the Moses-type PMGRR was used, list the criteria and equations used as described in the Technical Support Document available as part of the public record for this section.

(iii) *Phase 3 experiments: determination of the reaction quantum yield of the test chemical*—(A) *Treatment of results.* The objective of this set of experiments is to determine the reaction quantum yield for a specific test chemical $(\phi_c)$ at low optical density with a low optical density actinometer. The reaction quantum yield $\phi_c$ can be calculated using Equation 1 under paragraph (a)(3)(ii)(A)(*1*) of this section,

$$\phi_c=(k_{pc}/k_{pa})\ \epsilon_{\lambda a}/\epsilon_{\lambda c})\phi_a,$$

by the following steps.

(*1*) Photolysis experiments are carried out by simultaneously photolyzing the test chemical and actionometer in the PMGRR at the chosen wavelength λ. The concentration of test chemical and actinometer are measured periodically as a function of time. These data are then used to determine the ratio of the rate constants $(k_{pc}/k_{pa})$ employing linear regression analyses of the data on Equation 6 under paragraph (a)(3)(ii)(A) (*2*) of this section,

$$\ln(C_o/C_t)_c=(k_{pc}/k_{pa})\ln(C_o/C_t)_a,$$

with $\ln(C_o/C_t)_a$ as the independent variable and $\ln(C_o/C_t)_c$ as the dependent variable. The slope of the best straight line is the ratio of the rate constants $(k_{pc}/k_{pa})$. If a slight loss of test chemical or actinometer was detected in the controls at any time t, then employ the following procedure. Consider, as an example, the loss of test chemical in the control at time t. Using the average concentration of the test chemical in the controls from the replicates at time t and the average initial concentration, calculate $\ln(C_o/C_t)_c^{loss}$. Using the average concentration of test chemical from the replicates after photolysis time t, calculate $\ln(C_o/C_t)_c^{obs.}$. The corrected term is then

*Equation 27*

$$\ln(C_o/C_t)_c^{corr.}=\ln(C_o/C_t)_c^{obs.}-\ln(C_o/C_t)_c^{loss}.$$

The same procedure can be applied to obtain a corrected term for the actinometer. Using the corrected terms for test chemical and/or actinometer in Equation 6 under paragraph (a)(3)(ii)(A)(*2*) of this section, determine the ratio of the rate constants $(k_{pc}/k_{pa})$ as described under paragraph (c)(1)(iii)(A)(*1*) of this section.

(*2*) Determine the quantum yield of the actinometer $\phi_a$ using Equation 7 or 8 under paragraph (a)(3)(ii)(A)(*3*) of this section and the molar concentration of pyridine [PYR] present in the actinometer.

(*3*) Use the molar absorptivities of test chemical ($\epsilon_{\lambda_c}$) and actinometer ($\epsilon_{\lambda_a}$) at the wavelength the photolysis experiments were carried out (i.e., 313 or 366 nm).

(*4*) (*i*) Substitute the values of ($k_{pa}/k_{pc}$), $\epsilon_{\lambda_a}$, $\epsilon_{\lambda_c}$, and $\phi_a$ in Equation 1 under paragraph (a)(3)(ii)(A)(*1*) of this section and calculate the reaction quantum yield of the test chemical $\phi_c$.

(*ii*) A hypothetical example is presented in the paragraph (c)(3) of this section, to illustrate how all the data can be used in Procedure One to determine the reaction quantum yield of the test chemical and to determine ($k_{pE}$) and ($t_{1/2E}$) as a function of latitude and season of the year.

(B) *Other test conditions.* (*1*) Report the size, approximate cell wall thickness, the pathlength l, and type of glass used for tubes used to hold the test chemical and actinometer solutions.

(*2*) Report the initial pH of all test chemical solutions, if appropriate, and the type and concentration of the buffers employed for each pH.

(*3*) If acetonitrile was used to solubilize the test chemical, report the percent, by volume, of the acetonitrile which was used.

(*4*) If significant loss of test chemical occurred in the control solution, indicate the causes and how they were eliminated or minimized.

(C) *Test data report.* (*1*) Report the wavelength used in the photolysis experiments.

(*2*) Report the actinometer used.

(*3*) Report the initial molar concentration of test chemical $(C_o)_c$ of each replicate and the mean value.

(*4*) Report the initial molar concentration of actinometer $(C_o)_a$ and the molar concentration of pyridine used.

(*5*) For each time point, report the two separate values for the molar concentration of test chemical $(C_t)_c$ and actinometer $(C_t)_a$ and the mean values.

(*6*) For each time point, report the two separate values of the molar concentration of test chemical and actinometer controls and the mean values.

(*7*) Tabulate and report the following data: t, $\ln(C_o/C_t)_c$, and $\ln(C_o/C_t)_a$. From the linear regression analysis, report the ratio of the rate constants ($k_{pc}/k_{pa}$), and the correlation coefficient.

(*8*) If loss of test chemical and/or actinometer was observed during photolysis, then report the data $\ln(C_o/C_t)^{corr.}$, $\ln(C_o/C_t)^{obs.}$, and $\ln(C_o/C_t)^{loss}$ for the test chemical and/or actinometer for each time point. From the linear regression analysis of test chemical $\ln(C_o/C_t)_c^{corr.}$ and/or actinometer $\ln(C_o/C_t)_a^{corr.}$, report the ratio of the rate constants ($k_{pc}/k_{pa}$) and the correlation coefficient.

(*9*) Report the reaction quantum yield of the actinometer ($\phi_a$).

(*10*) Report the reaction quantum yield of the test chemical ($\phi_c$).

(*11*) Report $(k_{pE})_{max.}$, ($k_{pE}$), $(t_{1/2E})_{min.}$, and ($t_{1/2E}$) for the summer and winter solstices using the appropriate $L_\lambda$ values from § 796.3700(c)(3) Tables 3 to 6, closest to the latitude of the chemical manufacturing site.

(*12*) For chemicals that ionize or protonate, report the data for steps 1 through 11 for the experiments at the required pHs under paragraph (b)(1)(vi) of this section.

(2) *Procedure Two: Determination of the reaction quantum yield by the low optical density test chemical and high optical density actinometer method*—(i) *Phase 1 Experiments: UV-visible absorption spectra.* The treatment of results and data reporting are exactly the same as described in paragraph (c)(1)(i) of this section.

(ii) *Phase 2 experiments: Determination of the reaction quantum yield of the test chemical*—(A) *Treatment of results.* The objective of this set of experiments is to determine the reaction quantum yield of a test chemical ($\phi_c$) at low optical density with the high optical density ferrioxalate actinometer. $\phi_c$ can be calculated using Equation 9 under paragraph (a)(3)(ii)(B)(*1*) of this section),

$$\phi_c = \phi_a \, (k_{pc}/k_{pa}) \, (2.303\epsilon_{\lambda_c} l)^{-1},$$

by the following steps.

(*1*) In the first set of experiments, the ferrioxalate actinometer is photolyzed in a POB at the chosen wavelength λ (313 or 366 nm) and the molar concentration of ferrous ion $(C_t)_a$ is measured as a function of the time t. These data are fitted to Equation 11 under paragraph (a)(3)(ii)(B)(*1*) of this section using linear regression analysis. The slope is equal to $K_{pa}$. The data obtained from the third set of experiments are used in the same manner as described above to obtain another actinometer rate constant. These two rate constants are used to obtain an average actinometer rate constant $(k_{pa})_{ave.}$.

(*2*) In the second series of experiments, the test chemical is photolyzed in the same cell in the POB and the concentration of test chemical $(C_t)_c$ is measured as a function of the time t. These data are fitted to Equation 3 under paragraph (a)(3)(ii)(A)(*1*) of this section using linear regression analysis. The slope is equal to $k_{pc}$. If a slight loss of test chemical is detected in the controls, then follow the procedure outlined in paragraph (b)(1)(viii) of this section to calculate a corrected test chemical rate constant.

(*3*) Use the appropriate quantum yield of the ferrioxylate actinometer (at 0.15 M) at the chosen wavelength:

at 313 nm, $\phi_a=1.20$; at 366 nm, $\phi_a=1.18$.

(*4*) Use the molar absorptivity of the test chemical ($\epsilon_{\lambda c}$) at the appropriate photolysis wavelength (313 or 366 nm) determined in paragraph (c)(2)(i) of this section.

(*5*) Use the known pathlength of the reaction cell *l* under paragraph (b)(1)(iii) of this section.

(*6*) Substitute the values of $k_{pc}$, $(k_{pa})_{ave.}$, $\phi_a$, $\epsilon_{\lambda c}$, and *l* in Equation 9 under paragraph (a)(3)(ii)(B)(*1*) of this section and calculate the reaction quantum yield of the test chemical $\phi_c$.

(*7*) If the PMGRR equipment was used in these experiments, then repeat the calculations for Steps 1 through 6 obtained in this equipment.

(B) *Specific analytical and recovery procedures.* (*1*) Provide a detailed description or reference for the analytical procedures used, including the calibration data and precision; and

(*2*) If extraction methods were used to separate the solute from the aqueous solution, provide a description of the extraction method as well as the recovery data.

(C) *Other test conditions.* (*1*) Describe the equipment used (i.e., the type of PMGRR or POB). If a Moses-type PMGRR was used, report the aperture in $cm^2$.

(*2*) If the POB equipment was used, describe in detail the photolysis reaction cell. Report the volume, pathlength *l*, and a description of the optical windows used.

(*3*) If the PMGRR equipment was used, report the size, approximate cell wall thickness, and type of glass used for the reaction tubes.

(*4*) If the pathlength of the cell was measured by the procedure outlined in the Technical Support Document, available as part of the public record for this section, then report all the data obtained in determining the cell pathlength.

(*5*) If acetonitrile was used to solubilize the test chemical, report the percent by volume.

(*6*) Report the pH of all test chemical solutions, if appropriate.

(*7*) If a significant loss of test chemical occurred in the control samples, indicate the causes and how they were eliminated.

(D) *Test data report.* (*1*) Report the wavelength used in the photolysis experiments.

(*2*) For the actinometry experiments 1 and 3; (*i*) report for each time point the two values of the molar concentration of ferrous ion formed and the mean value; (*ii*) report the actinometer rate constant $k_{pa}$ and correlation coefficient for each experiment; and (*iii*) report the average actinometer rate constant $(k_{pa})_{ave.}$.

(*3*) For the test chemical photolysis experiment; (*i*) report the initial molar concentration of chemical $(C_o)_c$ of each replicate and the mean value; (*ii*) report the two separate values of the molar concentration of test chemical $(C_t)_c$ and the mean value at time t. Report the time t; (*iii*) report the two separate values of the molar concentration of the test chemical controls and the mean value for each time point. Report t for each time point; and (*iv*) report from the linear regression analyses of the data, the rate constant of the test chemical ($k_{pc}$), and the correlation coefficient.

(*4*) Report the reaction quantum yield of the actinometer and ($\phi_a$).

(*5*) Report the reaction quantum yield of the test chemical ($\phi_c$).

(*6*) Report $(k_{pE})_{max.}$, $(k_{pE})$, $(t_{1/2E})_{min.}$, and $(t_{1/2E})$ for the summer and winter solstices using the appropriate $L_\lambda$ values from § 796.3700(c)(3) Tables 3 to 6, closest to the latitude of the chemical manufacturing site.

(*7*) For chemicals that ionize or protonate, report the data for steps 1 through 6 for the experiments at the required pHs under paragraph (b)(1)(vi) of this section.

(3) *Hypothetical illustrative example: Determination of the reaction quantum yield by the low optical density test chemical and actinometer method and sunlight photolysis.* (i)(A) Consider a chemical plant just south of Peoria, Illinois on the Illinois River which produces an organic chemical A which is not an acid or a base. The waste effluent passes through a primary and secondary treatment plant and the waste, which still contains some chemical A, is then discharged into the river. The plant is located at 40.7 degrees north latitude. Information is needed on photolysis rates and half-lives of chemical A in aqueous media in the summer and winter seasons.

(B) The company research laboratory is located in Peoria, Illinois and the required photolysis data was needed in January. Since the temperature outdoors was well below freezing during this month, the outdoor sunlight photolysis experiments, described in § 796.3700 could not be carried out. Thus, it was necessary to carry out photolysis experiments in the laboratory. The research laboratory was equipped to carry out photolysis experiments with an Ace-type PMGRR and thus Procedure One was used.

(ii) Laboratory experiments, data, and calculations: (A) *Phase 1 experiments.* (*1*) Chemical A had a saturated water solubility of $3.9\times10^{-3}$ M at 25 °C. In the Phase 1 procedure, the UV-visible absorption spectrum was obtained for chemical A at a concentration of $1.00\times10^{-5}$ M in a 10.0 cm quartz cell in duplicate. Using the wavelength interval range from § 796.3700(c)(3) Table 1, the average absorbance of duplicate runs at λ center was obtained and the results are summarized in the following Table 1. (It must be emphasized that $\epsilon_{\lambda_c}$ has to be averaged over the wavelength intervals that correspond to the same intervals for the $L_\lambda$ values centered at λ. This has already been taken care of in this section since the wavelength interval ranges listed in Table 1 coincide with the same wavelength intervals for $L_\lambda$ centered at λ in § 796.3700(c)(3) Tables 3 to 6. In addition, the average absorbance was measured at 313.0 nm and at 366.0 nm and these results are summarized in the following Table 1:

TABLE 1—SUMMARY OF SPECTRAL AND PHOTOLYSIS DATA FOR CHEMICAL A

SPECTRAL DATA

| λ center (nm) | $A_{\lambda_c}$ | $\epsilon_{\lambda_c}(M^{-1}cm^{-1})$ |
|---|---|---|
| 297.5 | 0.5904 | 5904 |
| 300.0 | 0.5414 | 5414 |
| 302.5 | 0.4924 | 4924 |
| 305.0 | 0.4434 | 4434 |
| 307.5 | 0.3810 | 3810 |
| 310.0 | 0.3124 | 3124 |
| 312.5 | 0.2714 | 2714 |
| 315.0 | 0.2224 | 2224 |
| 317.5 | 0.2004 | 2004 |
| 320.0 | 0.1514 | 1514 |
| 323.1 | 0.1310 | 1310 |
| 330.1 | 0.1030 | 1030 |
| 340.0 | 0.0645 | 645 |
| 350.0 | 0.0473 | 473 |
| 360.0 | 0.0287 | 287 |
| 370.0 | 0.0170 | 170 |
| 380.0 | 0.0085 | 85 |
| 390.0 | 0.0030 | 30 |
| 400.0 | 0.0010 | 10 |
| 410.0 | 0.0000 | 0 |
| 313.0 | 0.2610 | 2610 |
| 366.0 | 0.0185 | 185 |

PHOTOLYSIS DATA

| λ center (nm) | Summer | | Winter | |
|---|---|---|---|---|
| | $L_\lambda$ [1] | $\epsilon_{\lambda_c}L_\lambda(day^{-1})$ | $L_\lambda$ [1] | $\epsilon_{\lambda_c}L_\lambda(day^{-1})$ |
| 297.5 | 6.17(−5) | 0.4 | 5.49(−7) | 0.0 |
| 300.0 | 2.70(−4) | 1.5 | 5.13(−6) | 0.0 |
| 302.5 | 8.30(−4) | 4.1 | 3.02(−5) | 0.2 |
| 305.0 | 1.96(−3) | 8.7 | 1.19(−4) | 0.5 |
| 307.5 | 3.74(−3) | 14.3 | 3.38(−4) | 1.3 |
| 310.0 | 6.17(−3) | 19.3 | 7.53(−4) | 2.4 |
| 312.5 | 9.07(−3) | 24.6 | 1.39(−3) | 3.8 |
| 315.0 | 1.22(−2) | 27.1 | 2.22(−3) | 4.9 |
| 317.5 | 1.55(−2) | 31.1 | 3.19(−3) | 6.4 |
| 320.0 | 1.87(−2) | 28.3 | 4.23(−3) | 6.4 |
| 323.1 | 3.35(−2) | 43.9 | 8.25(−3) | 10.8 |
| 330.0 | 1.16(−1) | 119.5 | 3.16(−2) | 32.6 |
| 340.0 | 1.46(−1) | 94.2 | 4.31(−2) | 27.8 |
| 350.0 | 1.62(−1) | 76.6 | 4.99(−2) | 23.6 |
| 360.0 | 1.79(−1) | 51.4 | 5.68(−2) | 16.3 |
| 370.0 | 1.91(−1) | 32.5 | 6.22(−2) | 10.6 |
| 380.0 | 2.04(−1) | 17.3 | 6.78(−2) | 5.8 |
| 390.0 | 1.93(−1) | 5.8 | 6.33(−2) | 1.9 |
| 400.0 | 2.78(−1) | 2.8 | 9.11(−2) | 0.9 |

PHOTOLYSIS DATA—Continued

| λ center (nm) | Summer |  | Winter |  |
|---|---|---|---|---|
|  | $L_\lambda$[1] | $\epsilon_{\lambda_c}L_\lambda$(day$^{-1}$) | $L_\lambda$[1] | $\epsilon_{\lambda_c}L_\lambda$(day$^{-1}$) |
| 410.0 | 3.64(−1) | 0.0 | 1.20(−1) | 0.0 |
|  |  | $\Sigma\epsilon_{\lambda_c}L_\lambda$=603.4 |  | $\Sigma\epsilon_{\lambda_c}L_\lambda$=156.2 |

[1] The units of $L_\lambda$ are in $10^{-3}$ einsteins cm$^{-2}$ day$^{-1}$. The second number in the columns in parenthesis is the power of ten by which the first number is multiplied.

From the Table 1 data and Equation 25 under paragraph (c)(1)(i)(A)(*1*) of this section, the average molar absorptivity is

*Equation 28*

$$\epsilon_{\lambda_c}=10^4A_{\lambda_c}.$$

From the average value of $A_{\lambda_c}$ at λ center, the average molar absorptivity can be obtained from Equation 28 and these results are summarized in Table 1, Spectral Data. In addition, using $A_{\lambda_c}$ at 313.0 and 366.0 nm in Equation 28, the molar absorptivity $\epsilon_{313_c}$ and $\epsilon_{366_c}$ can be obtained and these values are also given in Table 1, Spectral Data.

(*2*) Since the plant is located at 40.7 degrees north latitude, the closest $L_\lambda$ values are at 40 degrees north latitude. These values are obtained from § 796.3700(c)(3) Table 5 and are summarized in Table 1, Photolysis Data under paragraph (c)(3)(ii)(A)(*1*) of this section for the summer and winter solstices. Using the data from Table 1, Photolysis Data under paragraph (c)(3)(ii)(A)(*1*) of this section and Equations 16 and 17 under paragraph (a)(3)(ii)(B)(*6*) of this section, the following results are obtained.

| Summer solstice | Winter solstice |
|---|---|
| $(k_{pE})_{max.}=\Sigma\epsilon_{\lambda_c}L_\lambda=603$ days$^{-1}$.. | $(k_{pE})_{max.}=\Sigma\epsilon_{\lambda_c}L_\lambda=156$ days$^{-1}$ |
| $(t_{1/2E})_{min.}=1.1\times10^{-3}$ days ....... | $(t_{1/2E})_{min.}=4.4\times10^{-3}$ days. |

Since the chemical transforms rapidly on the summer and winter solstices, it is necessary to determine the reaction quantum yield of chemical A in the laboratory using Procedure One and to obtain direct sunlight photolysis rates and half-lives in aqueous media during the summer and winter seasons.

(B) *Phase 2 experiments—(1) Determination of the approximate rate constant of the test chemical.* (*i*) Chemical A was dissolved directly in pure water and a homogeneous solution was prepared. Analysis of duplicate samples indicated that the average concentration was $5.00\times10^{-6}$ M. Using the UV spectral data obtained, the absorbance at 313 nm in a one cm absorption cell containing a solution at a concentration of $5.00\times10^{-6}$ M was less than 0.02 [i.e., $A_{313_c}=2610\ (5.00\times10^{-6})(1.00)=0.0131$]. Hence, the test chemical solution in approximately 1 cm pathlength tubes was at low optical density.

(*ii*) A series of tubes (13×100 mm), with an effective pathlength of 1.12 cm, were filled with the aqueous solution of test chemical and sealed. Since $\epsilon_{313_c}>\epsilon_{366_c}$ in Table 1, under paragraph (c)(3)(ii)(A) (*1*) of this section, photolysis experiments were carried out at 313 nm in a PMGRR using the procedure described in paragraph (b)(2)(ii)(A) of this section. Duplicate photolyzed and control samples were removed periodically and analyzed for the concentration of test chemical. At t=39.4 hours, the average concentration of photolyzed sample $(C_t)_c$ was $2.25\times10^{-6}$ M and the average concentration of control sample was $4.99\times10^{-6}$ M. Thus, approximately 55 percent of the test chemical transformed. Since no loss of chemical was observed in the control sample, no adventitious processes occurred and the loss of chemical was only due to photolysis. Using Equation 3 under paragraph (a)(3)(ii)(A)(*1*) of this section and the data in this paragraph:

*Equation 29*

$$\ln(5.00\times10^{-6}/2.25\times10^{-6})=k_{pc}(39.4)$$

$$k_{pc}=0.0203\ \text{hours}^{-1}.$$

(*2*) *Actinometry experiments.* (*i*) Utilizing Equation 5 and the value of $k_{pc}$ obtained in Equation 29, the half-life of the test chemical is then

*Equation 30*

$$(t_{1/2})c=0.693/0.0203=34.1\ \text{hours}.$$

Since $(t_{1/2})c > 12$ hours, the PNAP/PYR actinometer is required for the photolysis experiments.

(*ii*) Using Equation 18 under paragraph (a)(3)(x)(A)(*1*)(*ii*) of this section and the approximate rate constant $k_{pe}$ from equation 29 under paragraph (c)(3)(ii)(B)(*1*)(*ii*) of this section, the concentration of pyridine needed to make the rate constant of the actinometer approximately equal to the rate constant of the test chemical is given below:

*Equation 31*

$$[PYR]=1.16\ k_{pe}=1.16(0.0203)$$
$$[PYR]=2.36\times10^{-3}\ M$$

An actinometer solution was then prepared according to the procedure given in paragraph (b)(2)(ii)(B)(*1*) of this section and the concentration of pyridine was $2.36\times10^{-3}$ M. The concentration of PNAP was measured in duplicate and the average concentration ($C_o$) was $0.900\times10^{-5}$ M. Using UV spectral data, the absorbance at 313 nm in a one cm absorption cell containing an actinometer solution at a concentration of $0.900\times10^{-5}$ M was less than 0.02 [i.e., $A_{313_a}=(2056)\ (0.900\times10^{-5})\ (1.00)=0.0185$]. Hence, the actinometer solution in approximately 1 cm pathlength tubes was at low optical density.

(*iii*) A series of tubes, identical to those used in the test chemical photolysis experiments, were filled with the actinometer solution and sealed. The photolysis experiments were carried out in the same PMGRR at 313 nm using the procedure described in paragraph (b)(2)(ii)(B)(*2*) of this section. Duplicate photolyzed and control samples were periodically removed and analyzed for the concentration of PNAP. At 50.6 hours, the average concentration of photolyzed sample $(C_t)_a$ was $0.360\times10^{-5}$ M and the average concentration of the control sample was $0.900\times10^{-5}$ M. Thus, approximately 60 percent of PNAP transformed. Since no loss of chemical was observed in the control sample, no adventitious processes occurred and the loss of chemical was only due to photolysis. Using equation 2 under paragraph (a)(3)(ii)(A)(*1*) of this section and the data:

*Equation 32*

$$\ln(0.900\times10^{-5}/0.360\times10^{-5})=k_{pa}\ (50.6)$$
$$k_{pa}=0.0181\ \text{hours}^{-1}.$$

Thus, at the molar concentration of pyridine given by equation 31, $k_{pa}$ is approximately the same as $k_{pe}$ (within ± 50 percent).

(C) *Phase 3 experiments.* (*1*) A solution of test chemical was prepared according to the procedures described in paragraph (b)(2)(ii)(A)(*2*) of this section and the concentration $(C_o)_c$ was measured in duplicate and the average concentration was found to be $5.00\times10^{-6}$ M. An actinometer solution (PNAP/PYR) was prepared according to the procedure described in paragraph (b)(2)(ii)(B)(*1*) of this section with a pryridine concentration of $2.36\times10^{-3}$ M under Equation 31 in paragraph (c)(3)(B)(*2*)(*ii*) of this section. The average concentration of PNAP $(C_o)_a$ from duplicate samples was measured and found to be $9.00\times10^{-6}$ M. These solutions were placed in identical tubes (13×100 mm), sealed, and photolyzed at 313 nm in the PMGRR according to the procedure given in paragraph (b)(2)(iii) of this section. The average concentration of duplicate samples of test chemical, test chemical control, actinometer (PNAP), and actinometer control, obtained in this photolysis experiment, is summarized in the following Table 2:

TABLE 2—PHOTOLYSIS DATA FOR TEST CHEMICAL A AND (PNAP/PYR) ACTINOMETER

| t(hours) | Average test chemical concentration $\times10^{6}$ | | Average PNAP concentration $\times10^{6}$ | |
|---|---|---|---|---|
| | Photolyzed samples | Controls | Photolyzed samples | Controls |
| 0 | 5.00 | 5.00 | 9.00 | 9.00 |
| 12 | 3.92 | 4.98 | 7.24 | 8.99 |
| 24 | 3.07 | 5.00 | 5.83 | 8.98 |
| 36 | 2.41 | 4.99 | 4.60 | 9.00 |
| 48 | 1.89 | 4.98 | 3.78 | 8.98 |
| 60 | 1.48 | 4.99 | 3.04 | 8.99 |
| 72 | 1.16 | 5.00 | 2.44 | 9.00 |

(*2*)(*i*) Since no significant loss of PNAP or test chemical was observed in the control samples, no adventitious processes occurred and the loss of test chemical and PNAP was only due to photolysis. Utilizing the data in under paragraph (c)(3)(ii)(C)(*1*) Table 2, of this section ln $(C_o/C_t)$ for the test chemical and actinometer solution can be calculated and the results are summarized in the following Table 3:

TABLE 3—PHOTOLYSIS DATA FOR TEST CHEMICAL AND ACTINOMETER (PNAP/PYR)

| t (hours) | Test chemical |  | Actinometer (PNAP) |  |
|---|---|---|---|---|
|  | $(C_t)_c \times 10^{5}$ (M) | $\ln(C_o/C_t)_c$ | $(C_t)_a \times 10^{5}$ (M) | $\ln(C_o/C_t)_a$ |
| 0 | 5.00 | 0.000 | 9.00 | 0.000 |
| 12 | 3.92 | 0.243 | 7.24 | 0.218 |
| 24 | 3.07 | 0.488 | 5.83 | 0.434 |
| 36 | 2.41 | 0.730 | 4.69 | 0.652 |
| 48 | 1.89 | 0.973 | 3.78 | 0.868 |
| 60 | 1.48 | 1.22 | 3.04 | 1.09 |
| 72 | 1.16 | 1.46 | 2.44 | 1.31 |

(*ii*) The ratio of the rate constants $(k_{pc}/k_{pa})$ is defined by Equation 6 under paragraph (a)(3)(ii)(A)(*2*) of this section,

$$\ln(C_o/C_t)_c = (k_{pc}/k_{pa}) \ln(C_o/C_t)_a.$$

Utilizing all the data in paragraph (c)(3)(ii)(C)(*2*)(*i*) Table 3 of this section including the time point $t=0$ and linear regression analysis, the slope is found to be 1.12 with a correlation coefficient of 1.000.

(*iii*) The quantum yield of the PNAP/PYR actinometer is given by Equation 7 under paragraph (a)(3)(ii)(A)(*3*) of this section,

$$\phi_a = 0.0169\ [\text{PYR}].$$

Since the pyridine concentration in the actinometer was $2.36 \times 10^{-2}$ M under Equation 31 in paragraph (c)(3)(B)(*2*)(*ii*) of this section, the quantum yield of the actinometer $(\phi_a)$ is $3.99 \times 10^{-4}$.

(*3*) The reaction quantum yield of the test chemical is given by Equation 1 under paragraph (a)(3)(ii)(A)(*1*) of this section,

$$\phi_c = (k_{pc}/k_{pa})\ (\epsilon_{\lambda_a}/\epsilon_{\lambda_c})\ \phi a.$$

The pertinent data are summarized as follows:

$\phi_a = 3.99 \times 10^{-4}$, $(k_{pc}/k_{pa}) = 1.12$, $\epsilon_{\lambda_c} = 2610$, and $\epsilon_{\lambda_a} = 2056$.

Substituting these data in Equation 1 under paragraph (a)(3)(ii)(A)(*1*) of this section yields

*Equation 33*

$$\phi_c = (1.12)\ (2056/2610)\ (3.99 \times 10^{-4})$$

$$\phi_c = 3.52 \times 10^{-4}$$

(*4*) The rate constants for direct photolysis of test chemical in aqueous media and the half-life for water bodies and clear sky conditions for the winter and summer seasons can be calculated as follows: The values of $\Sigma\epsilon_{\lambda_c} L_\lambda$ have been calculated for the summer and winter solstices under paragraph (c)(3)(ii)(A)(*1*) Table 1, Photolysis Data of this section. For summer $\Sigma\epsilon_{\lambda_c} L_\lambda = 603$ days $^{-1}$; for winter $\Sigma\epsilon_{\lambda_c} L_\lambda = 156$ days $^{-1}$. The reaction quantum yield for chemical A is $3.52 \times 10^{-4}$ Equation 33 under paragraph (c)(3)(B)(*3*) of this section. Using these data in Equation 16 under paragraph (a)(3)(B)(*6*) of this section yields

*Equation 34 (summer)*

$$k_{pE} = 3.52 \times 10^{-4} (603) = 0.213 \text{ days}^{-1}$$

*Equation 35 (winter)*

$$k_{pE} = 3.52 \times 10^{-4} (156) = 0.0549 \text{ days}^{-1}.$$

These values can be substituted into Equation 18 under paragraph (b)(1)(x)(A)(*1*)(*ii*) of this section to obtain the half-lives for these two seasons.

*Equation 36 (summer)*

$$(t_{1/2E})_c = 0.693/0.213 = 3.3 \text{ days}$$

*Equation 37 (winter)*

$$(t_{1/2E})_c = 0.693/0.0549 = 13 \text{ days}.$$

These results are valid for clear-sky conditions and at shallow depths in the Illinois River.

(4) *Glossary of important symbols.*

$\lambda$—Wavelength $\lambda$.
$A_\lambda$—Absorbance at wavelength $\lambda$.
$F_{s\lambda}$—Fraction of light absorbed by the system at wavelength $\lambda$.
$F_{c\lambda}$—Fraction of light absorbed by a chemical (c) at wavelength $\lambda$.
$\epsilon\lambda c$—Molar absorptivity of a chemical (c) at wavelength $\lambda$.
$\epsilon\lambda a$—Molar absorptivity of an actinometer (a) at wavelength $\lambda$.
$\alpha\lambda$—Absorption (or attenuation) coefficient of water at wavelength $\lambda$.
l—The light pathlength; the distance traveled by a beam of light passing through the system.
$\phi_a$-Reaction quantum yield of an aqueous solution of actinometer (a).
$\phi_c$-Reaction quantum yield of an aqueous solution of chemical (c).
$\phi_E$—Sunlight reaction quantum yield of a chemical (c) in a water body in the environment.

-d[C]/dt—Direct photolysis rate of chemical (c).

$k_{pE}$—Direct photolysis sunlight rate constant in a water body in the environment.

$(k_{pE})_{max.}$—Maximum direct photolysis rate constant in a water body in the environment.

$k_{pc}$—Direct photolysis rate constant of constant of chemical (c) in water measured in the laboratory.

$k_{pa}$—Direct photolysis rate constant of actinometer (a) in water measured in the laboratory.

$k_{a\lambda}$-Specific light absorption of a photoreactive chemical at low concentration and at wavelength λ.

$k_a$—Specific light absorption rate constant integrated over all wavelengths absorbed by the chemical.

$(t_{1/2})c$—Half-life of a chemical (c) in water.

$(t_{1/2})a$—Half-life of an actinometer (a) in water.

$t_{1/2E}$—Half-life of a chemical in a water body in sunlight in the environment.

$(t_{1/2E})_{min.}$—The minimum sunlight half-life of a chemical in a water body in sunlight in the environment.

$I_{o\lambda}$-The number of photons (or einsteins) of light of wavelength λ in the system per $cm^2$ per second.

$I_\lambda$-The number of einsteins of light of wavelength λ in the system per liter per hour (or second).

$L_\lambda$—Solar irradiance in a water body at shallow depths in the units milli einsteins per $cm^2$ per second.

PYR—Pyridine.

[PYR]—Molar concentration of pyridine.

PNAP—p-Nitroacetophenone.

PNA—p-Nitroanisole.

o-NB—o-Nitrobenzaldehyde

PNAP/PYR—p-Nitroacetophenone-pyridine actinometer.

PNA/PYR—p-Nitroanisole-pyridine actinometer.

POB—Photochemical optical bench.

PMGRR—Photochemical "Merry-Go-Round" Reactor.

$A_p$—Aperture in the PMGRR.

(d) *References.* For additional background information on this test guideline the following references should be consulted:

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**§ 796.3800 Gas phase absorption spectra and photolysis.**

(a) *Introduction*—(1) *Background and purpose.* Numerous chemicals enter the atmosphere from a variety of sources. For example, chemicals enter the atmosphere as a result of the burning of coal, from the combustion of gasoline in cars and diesel fuel in trucks, and from the release of volatile organic chemicals during manufacture, processing, use, and disposal. Pesticides, applied from airplanes, enter the atmosphere directly and volatilize from soils and water bodies. Chemical pollutants present in the atmosphere can undergo photochemical transformation in the environment by direct photolysis in sunlight. Quantitative data in the form of rate constants and half-lives are needed to determine the importance of direct photolysis of pollutants in the atmosphere. This test method describes a first-tier screening level test method to estimate the maximum direct photolysis rate constant and minimum half-life of chemicals in the atmosphere in sunlight as a function of latitude and season of the year in the United States.

(2) *Definitions and units.* (i) "Radiant energy," or radiation, is defined as the energy traveling as a wave unaccompanied by transfer of matter. Examples include x-rays, visible light, ultraviolet light, radio waves, etc.

(ii) "Absorbance ($A_\lambda$)" is defined as the logarithm to the base 10 of the ratio of the initial intensity ($I_o$) of a beam of radiant energy to the intensity (I) of the same beam after passage through a sample at a fixed wavelength λ. Thus, $A_\lambda = \log_{10}(I_o/I)$.

(iii) The "Beer-Lambert law" states that the absorbance of a chemical in the gas phase, at a fixed wavelength, is proportional to the thickness of the absorbing material (l), or the light pathlength, and the concentration of the absorbing species (C).

(iv) "Cross section ($\sigma_\lambda'$)" is defined as the proportionality constant in the Beer-Lambert law. Thus, $A_\lambda = \sigma_\lambda' Cl$, where $A_\lambda$ is the absorbance, C is the concentration in molecules per $cm^3$ and $l$ is the pathlength in cm. The units of the cross section $\sigma_\lambda'$ are $cm^2$ $molecule^{-1}$. Numerical values of the cross section depend upon the nature of the absorbing species.

(v) A "first-order reaction" is defined as a reaction in which the rate of disappearance of a chemical is directly proportional to the concentration of the chemical and is not a function of the concentration of any other chemical present in the reaction mixture.

(vi) The "half-life ($t_{1/2}$)" of a chemical is defined as the time required for the concentration of the chemical being photolyzed to be reduced to one-half its initial value.

(vii) The "sunlight direct potolysis rate constant ($k_{pE}$)" is the first-order rate constant in the units of $day^{-1}$ and is a measure of the rate of disappearance of a chemical in the gas phase in sunlight.

(viii) The "actinic solar irradiance in the atmosphere ($J_\lambda$)" is related to the sunlight intensity in the atmosphere and is proportional to the average light flux (in the units of photons $cm^{-2}$ $day^{-1}$) that is available to cause photoreaction in the wavelength interval Δλ, centered at λ, over a 24-hour day at a specific latitude and season date. It is the irradiance which would be measured by a weakly absorbing spherical actinometer exposed to direct solar radiation and sky radiation from all directions.

(ix) "The Grotthus-Draper law," the first law of photochemistry, states that only light which is absorbed can be effective in producing a chemical transformation.

(x) The "Stark-Einstein law," the second law of photochemistry, states that only one molecule is activated to an excited state per photon or quantum of light absorbed.

(xi) The "reaction quantum yield ($\phi_\lambda$)" for an excited state process is defined as the fraction of absorbed light that results in photoreaction at a fixed wavelength λ. It is the ratio of a number of molecules that photoreact to the number of quanta of light absorbed or the ratio of the number of moles that photoreact to the number of einsteins of light absorbed at a fixed wavelength λ.

(xii) "Direct photolysis" is defined as the direct absorption of light by a chemical followed by a reaction which transforms the parent chemical into one or more products.

(3) *Principle of the test method.* (i) For weak absorbance of a chemical in the atmosphere, the first-order direct photolysis rate constant, $k_{pE}$, is given by the equation

*Equation 1*

$$k_{pE}=2.30\Sigma\phi_\lambda\sigma'_\lambda J_\lambda.$$

Where $\phi_\lambda$ is the reaction quantum yield; $\phi'_\lambda$ is the cross section in the units $cm^2$ $molecule^{-1}$ averaged over a wavelength interval $\Delta\lambda$, centered at $\lambda$; $J_\lambda$ is the actinic solar irradiance in the units photons $cm^{-2}$ $day^{-1}$ averaged over the wavelength interval $\Delta\lambda$, centered at $\lambda$; and the summation is taken over the range $\Delta\lambda=290$ to 800 nm. $J_\lambda$ is the solar actinic irradiance in the atmosphere under clear sky conditions and is a function of latitude and season of the year.

(ii) Since this photolysis process is first-order, the half-life ($t_{1/2}$) of a chemical is then given by

*Equation 2*

$$t_{1/2}=0.693/k_{pE}$$

(iii) A simple first-tier screening test has been developed using Equation 1. As an approximation, it is assumed that the reaction quantum yield $\phi_\lambda$ is equal to one, the maximum value. As a result, the upper limit for the direct photolysis sunlight rate constant in the gas phase is obtained and Equation 1 becomes

*Equation 3*

$$(k_{pE})_{max}=2.30\Sigma\sigma'_\lambda J_\lambda.$$

Using Equation 3 in Equation 2, the lower limit for the half-life is then given by

*Equation 4*

$$(t_{1/2})_{min}=0.693/(k_{pE})_{max}.$$

The cross section can be determined experimentally by the procedures outlined in paragraph (b) of this section and the values of $J_\lambda$ are given in Tables 1 to 4 under paragraph (c)(3) of this section as a function of latitude and season of the year in the United States. These data can then be used in Equation 3 to calculate $[k_{pE}]_{max}$. Finally, $(k_{pE})_{max}$ can be substituted in Equation 4 to calculate $[t_{1/2}]_{min}$.

(4) *Applicability and specificity.* (i) This test method is applicable to all chemicals which have UV-visible absorptions in the range 290 to 800 nm. Some chemicals only have absorptions below 290 nm and consequently cannot undergo direct photolysis in sunlight (e.g., chemicals such as alkanes, alkenes, alkynes, dienes, and fluoroalkanes).

(ii) This test method is only applicable to pure chemicals and not to the technical grade.

(iii) The first-tier screening test can be employed to estimate $(k_{pE})_{max}$ and $(t_{1/2E})_{min}$. If these data indicate that gas phase photolysis is an important process relative to other gas phase transformation processes (e.g., oxidation with hydroxyl radicals or ozone), then it is recommended that an upper-tier photolysis test be carried out to determine the reaction quantum yield and thus obtain more precise environmentally relevant rate constants and half-lives in sunlight. The data obtained from this first-tier test method can be used to determine $(k_{pE})_{max}$ for a test chemical as a function of latitude and season of the year in the United States under clear sky conditions. These rate constants are in a form suitable for preliminary mathematical modeling for environmental fate of a test chemical.

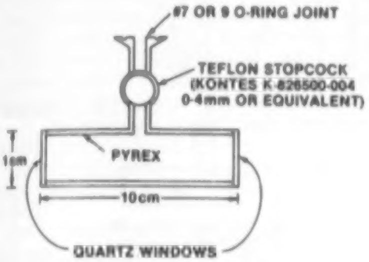
(b) *Test procedures.* The procedures outlined in this test method are based on the method proposed by Mill et al. (1982) under paragraph (d)(1) of this section and developed by Pitts et al. (1981) under paragraph (d)(2) of this section. It is also recommended that § 796.1050. *Absorption in Aqueous Solution: Ultraviolet/Visible Spectra*, be consulted for additional guidance.

(1) *Test conditions*—(i) *Ultraviolet-visible spectrophotometer.* Although single-beam spectrophotometers may be used, recording double beam spectrophotometers are highly recommended. It is extremely important that the spectrophotometer be able to scan over the wavelength region 270 to 800 nm and have an absorbance sensitivity, at a signal/noise ratio of one, of approximately 0.001. It is important that the spectrophotometer be able to attain a 90 percent separation of two monochromatic spectral features ap-

proximately 4 nm apart, peak to peak (i.e., the resolution should be at least 4 nm). It is also desirable to have a spectrophotometer that can accommodate absorption cells of length >10 cm. A Cary 219 UV-Visible Spectrophotometer, or an equivalent model, is highly recommended.

(ii) *Vapor and liquid absorption cells.* (A) Long pathlength cells are preferable; however, many commercial spectrophotometers will only accept absorption cells of 10 cm or less. A suitable vapor cell is depicted in the following Figure 1.

FIGURE 1—GAS ABSORPTION CELL

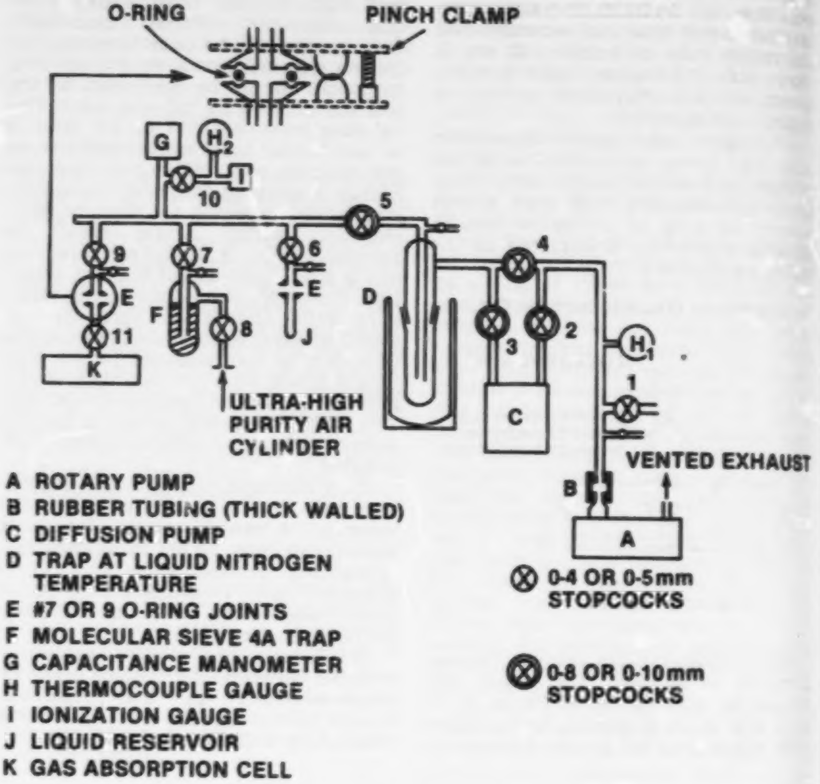


(B) A suitable vapor cell can be constructed as follows. The vapor cell should be constructed of Pyrex, 1 cm O.D. and 10 cm in length, and be fitted with plane parallel quartz windows at each end. The quartz windows can be conveniently attached to the Pyrex cell with vaccum tight epoxy resin (e.g., Torr-Seal, Varian Associates) only applied to the outside surface. A Teflon stopcock (or a Pyrex "o" ring stopcock) should be connected to the cell and contain an "o"-ring joint. The "o"-ring joint (e.g., #7 or #9, Kontes or Ace Glass) must match the one on the vacuum rack. Viton "o"-rings are highly recommended and should be frequently inspected for signs of deterioration which would result in vacuum leaks. A matched reference cell is extremely useful but not essential. However, the sample and reference cells should be very similar. Small spectral differences between the cells can be compensated for by running a blank with the sample and reference cells in the spectrophotometer. The use of stopcock grease is not required with these cells and should be avoided.

(C) A matched pair of liquid absorption cells is very desirable but is not essential. A pair of quartz ultraviolet absorption cells, 10 cm in length, and containing ground glass or Teflon stoppers are recommended. These liquid absorption cells are readily available commercially.

(iii) *Vacuum gas handling system.* A suitable gas handling system is shown diagramatically in the following Figure 2 and should be constructed completely with Pyrex glass.

FIGURE 2—SCHEMATIC OF GAS HANDLING VACUUM RACK


O-RING
PINCH CLAMP
G
H2
I
10
5
9
7
6
E
E
F
8
J
D
4
3
2
H1
1
11
K
ULTRA-HIGH PURITY AIR CYLINDER
C
VENTED EXHAUST
B
A
A ROTARY PUMP
B RUBBER TUBING (THICK WALLED)
C DIFFUSION PUMP
D TRAP AT LIQUID NITROGEN TEMPERATURE
E #7 OR 9 O-RING JOINTS
F MOLECULAR SIEVE 4A TRAP
G CAPACITANCE MANOMETER
H THERMOCOUPLE GAUGE
I IONIZATION GAUGE
J LIQUID RESERVOIR
K GAS ABSORPTION CELL
0-4 OR 0-5mm STOPCOCKS
0-8 OR 0-10mm STOPCOCKS

The components of the gas handling system are discussed below. The use of stopcock grease is not required and should be avoided.

(A) *Vacuum pumping system.* (*1*) In order to achieve a good vacuum, i.e., pressures $<10^{-5}$ torr ($1.3\times10^{-6}$ kPa), two pumps are required. The forepump (A) must be capable of achieving a pressure <0.05 torr (0.0065 kPa). A rotary pump (e.g., a Welch Model 1402 Duo-Seal or an equivalent model) is recommended. The forepump can be attached to the vacuum system by means of heavy-walled rubber vacuum tubing (B), or any flexible vacuum tubing. The exhaust from this pump should be vented into a hood.

(*2*) The second pump, a high vacuum model, should be a multistage oil diffusion pump (C) [e.g., a Consolidated Vacuum Corp. VMF-10 or VMF-20 or an equivalent model]. The pump fluid should be a silicone oil with a room temperature vapor pressure of $<10^{-6}$ torr ($1.3\times10^{-7}$ kPa) [e.g., Dow-Corning D.C. 702 or 703, or an equivalent grade].

(*3*) It is extremely important that the pumping system contain a trap (D) cooled with liquid nitrogen. The cone and socket joint on this trap can be conveniently sealed with Apiezon W wax, or an equivalent grade. This wax requires only gentle heating to apply and makes an effective vacuum seal. It is possible that a few test chemicals could dissolve Apiezon W wax. In this case, an inert silicone grease may be used to seal the trap.

(B) *Vacuum rack.* The recommended vacuum rack assembly is depicted in Figure 2 under paragraph (b)(1)(iii) of this section. All stopcocks should be of Teflon with Viton "o"-rings [Kontes K-826500 or K-826510 series or equivalent grades (or Pyrex "o" ring stopcocks)]. The "o"-ring joints (E) [#7 or #9] must be compatible with those on the gas absorption cell (K) or on the liquid reservoir (J). These "o"-ring joints should be clamped by pinch clamps with a screw lock device (e.g., Thomas #18A, or an equivalent grade).

(C) *Pressure Gauges.* Three pressure gauges are required:

(*1*) An ionization gauge to measure high vacuum [$<10^{-3}$ torr ($1.3\times10^{-4}$ kPa)].

(*2*) A thermocouple gauge to monitor the pressure in the range $10^{-3}$ to 1 torr ($1.3\times10^{-4}$ to 0.13 kPa). A convenient pressure monitoring system which contains ionization and thermocouple gauges is a Consolidated Vacuum Corp. Model GIC-300A or an equivalent model.

(*3*) A pressure gauge to monitor the pressure of the test chemical and diluent in the range 0.01 to 760 torr (0.0013 to 101.3 kPa); for example, an MKS Baratron 310 BHS-1000 with the associated 170-6C electronics unit and a digital readout or an equivalent model. While this vacuum gauge exhibits a slow zero drift, it can be readily rezeroed using the ionization gauge, i.e., when the ionization gauge reads approximately $10^{-3}$ torr (0.00013 kPa) or less.

(2) *Operation of the gas handling system.* Since there are a wide variety of procedures available for operating a gas handling system, the method used is left to the discretion of the tester. For those testers who do not have experience in handling a vacuum system, the detailed procedure described in paragraph (c)(5) of this section is highly recommended.

(3) *Preparation of samples*—(i) *Preparation of the gas phase test chemical sample: Preliminary Steps.* (A) If the test chemical is a gas at room temperature, then attach the gas container to the "o"-ring at the point where the liquid reservoir (J) is placed. Close stopcocks 2 and 3 and open 4. Pump until the pressure is less than $10^{-2}$ torr ($1.3\times10^{-3}$ kPa) as read on thermocouple gauge ($H_2$). Then open stopcocks 2 and 3 and close 4 and pump until the pressure is less than $10^{-5}$ torr ($1.3\times10^{-6}$ kPa) as read on the ionization gauge (I).

(B) If the test chemical is a liquid at room temperature, add a few cm$^3$ of liquid to a reservoir tube (J), sealed at one end and containing an "o"-ring at the other end, and connect the tube via the "o"-ring to stopcock 6. Freeze the sample with a Dewar containing liquid nitrogen, close stopcocks 2 and 3 and open 4 and 6. Degas the test chemical by allowing it to warm up to the liquid state, briefly degas, and refreeze the liquid. Repeat this process three or more times until the evolution of gas bubbles ceases upon thawing. Freeze the liquid, open stopcocks 2 and 3 and close 4. Pump until the pressure is less than $10^{-5}$ torr ($1.3\times10^{-6}$ kPa) as indicated by the ionization gauge (I). Close stopcock 6.

(ii) *Introduction of the test chemical into the gas absorption cell.* (A) For introduction of the test chemical into the gas absorption cell, close stopcocks 5, 7 and 10, with 9 and 11 open. If the test chemical is a gas, then stopcock 6 should be opened and the gas container valve is gradually opened to admit the gas into the gas handling manifold and gas absorption cell until the desired pressure is attained, as read on the capacitance manometer (G). Close the gas container valve and stopcock 6 and allow approximately 5 minutes before the final pressure at (G) is read. If the pressure has not stabilized in approximately 5 minutes, allow the cell to condition for several hours before the final pressure at (G) is read.

(B) For a liquid chemical in the reservoir (J), which has been degassed and is at liquid nitrogen temperature, the liquid nitrogen Dewar should be removed and stopcock 6 opened. The cold liquid in the reservoir (J) is allowed to warm up until the required pressure is attained, as read by the capacitance manometer (G). Close stopcock 6 and cool the reservoir again with liquid nitrogen and allow approximately 5 minutes before the final pressure at (G) is read. If the pressure has not stabilized in approximately 5 minutes, allow the cell to condition for several hours before the final pressure at (G) is read.

(C) With stopcocks 6, 8 and 11 closed and 5, 7, 9, and 10 open, the gas handling manifold is evacuated as described previously to a pressure less than $10^{-5}$ torr ($1.3\times10^{-6}$ kPa). Stopcocks 5 and 10 are then closed and ultra-high purity air from a cylinder is admitted into the gas handling manifold via stopcock 8 and through the trap (F) containing Molecular Sieve 4A. When the manifold is at one atmosphere pressure, as measured by pressure gauge (G), stopcock 11 is briefly opened to pressure the gas absorption cell to one atmosphere, and then closed. Stopcocks 8 and 9 are closed and the gas handling system is evacuated as described previously. The gas absorption cell can then be removed from (E) and covered to avoid photolysis.

(D) Based on the pressure P of the test chemical, as measured by gauge (G), the concentration of the gas sample is

*Equation 5*

$$C\ (\text{molecules cm}^{-3}) = 9.657\times10^{18}\ P(\text{torr})/T(K)$$

*Equation 5a*

$$C\ (\text{molecules cm}^{-3}) = 1.287\times10^{19}\ P(\text{kPa})/T(K).$$

where T is the room temperature in °K, which should be routinely monitored with a thermometer.

(E) The recommended pressure of the test chemical should be in the range 1-5 torr (0.13-0.65 kPa) where the Beer-Lambert law is obeyed. A final check on whether the test chemical obeys the Beer-Lambert law can be accomplished by demonstrating the constancy of the cross section at three partial pressures differing by a factor of 10.

(iii) *Preparation of solution phase test chemical sample.* (A) If the properties of the test chemical (i.e., small cross sections, low vapor pressure) are such that the maximum absorbance obtainable is one-tenth of the most sensitive spectrophotometer scale or less (i.e., $<0.001$ absorbance), a solution-phase study should be undertaken. The most sensitive scale may be limited by inherent spectrophotometer noise. For example, a given spectrophotometer's most sensitive scale is 0.00 to 0.10 absorbance units. Therefore, a test chemical for which the product of its maximum cross section and its concentration is less than 0.001 (in a 10 cm cell) could not be analyzed in the vapor phase with this particular spectrophotometer.

(B) The following spectroscopic grade chemicals are recommended to prepare solutions: chloroform, n-hexane, acetonitrile, and cyclohexane. Solutions of up to 10 percent by volume of test chemical can be prepared in one of these solvents in the standard manner.

(C) The concentration of the test chemical is given by the equations

*Equation 6*

$$C\ (\text{molecules cm}^{-3}) = 6.022\times10^{23}\ \text{mass (gms)}/\text{fw}\ (V_d)$$

*Equation 6a*

$$C\ (\text{molecules cm}^{-3}) = 6.022\times10^{23}\ V_s\rho/\text{FW}\ (V_d).$$

where $V_s$ is the volume of test chemical delivered into a volume $V_d$ of solvent in $cm^3$, fw is the formula weight of the test chemical in gms, and $\rho$ is the density of the test chemical in gms $cm^{-3}$ at the room temperature the solution was prepared.

(4) *Procedure for obtaining the spectrum.* As a general guide to obtaining UV-visible absorption spectra, the procedures outlined in § 796.1050 *Absorption in Aqueous Solution: Ultraviolet/Visible Spectra*, are highly recommended. Since the method presented in this procedure was developed by Pitts et al. (1981), it is highly recommended that this report be consulted for further details.

(i) *Determination of the cell pathlength.* The method for determining the cell pathlength of gas or liquid cells is left to the discretion of the tester. However, the method listed in § 796.1050, using one of their reference compounds, is highly recommended.

(ii) *Gas Phase Spectrum.* Measure the absorbance of the test chemical in duplicate relative to a matched cell filled with ultra-high purity air from the same cylinder similarly passed through trap (F) containing the molecular sieve. The absorbance should be measured at wavelengths $\lambda > 280$ nm using minimum slit widths. Record, in duplicate, the baseline when both the same reference cells are filled with high purity air dried through the molecular sieve and at the same settings as used for the test chemical sample. These data will be used to calculate the cross section, $\sigma'_\lambda$, at the appropriate wavelength intervals, centered at wavelength $\lambda$, listed in Tables 1 through 4, under paragraph (c)(3) of this section.

(iii) *Solution Phase Spectrum.* (A) Measure the absorbance of the test chemical in duplicate relative to a matched cell containing the solvent. The absorbance should be measured for wavelengths $\lambda > 280$ nm using the minimum slit widths. Record, in duplicate, the baseline when both the sample and reference cells are filled with the solvents. These data will be used to calculate the cross sections, $\sigma'_\lambda$, for the appropriate wavelength intervals, centered at $\lambda$, listed in Tables 1 through 4 under paragraph (c)(3) of this section.

(B) The concentration of the test chemical should be in the range where the Beer-Lambert law is obeyed. A check on whether the test chemical obeys this law can be accomplished by demonstrating the constancy of the cross section at 3 concentrations differing by a factor of 10.

(c) *Data and reporting*—(1) *Treatment of results*—(i) *Determination of the cross section from the gas phase spectrum.* (A) The cross section, $\sigma_\lambda$, can be determined from the gas phase absorption spectrum and the Beer-Lambert law in the form

*Equation 7*

$$\sigma'_\lambda = A_\lambda / Cl$$

where $A_\lambda$ is the absorbance at wavelength $\lambda$, centered in the wavelength interval $\Delta\lambda$, C is the concentration of test chemical in molecules $cm^{-3}$, and $l$ is the cell pathlength in cm. The cross section of the test chemical should be determined for the wavelength intervals listed in Tables 1 through 4 under paragraph (c)(3) of this section.

(B) There are at least three nondestructive methods of determining the absorbance over a specified wavelength interval: estimation, square counting, and planimetry. For many spectra, estimating an average absorbance over a small wavelength interval is sufficient to yield accurate results. However, for spectra containing rapidly changing absorptions and complex fine structure, square counting or planimetry should be used. These two methods require the integration of a definite region (in $A_\lambda$ x nm) followed by division by the width of the region in nm to obtain absorbance. The method using a compensating polar planimeter is the most accurate and is highly recommended. The absorbance should be obtained from the average of three tracings.

(ii) *Determination of the cross section from the solution phase spectrum.* The cross section, $\sigma'_\lambda$, can be determined from the solution phase spectrum using Equation 7 for the wavelength intervals listed in Tables 1 through 4 under paragraph (c)(3) of this section. For solution spectra, estimating an average absorbance over the wavelength intervals is sufficient to yield accurate results.

(iii) *Estimation of the maximum direct photolysis rate constant and minimum half-life in the gas phase.* (A) Using the cross sections obtained from the spectra and the values of $J_\lambda$ from Tables 1-4 under paragraph (c)(3) of this section, the maximum direct photolysis rate constant $(k_{pE})_{max}$ can be calculated at a specific latitude and season for the year using Equation 3. The minimum half-life, $(t_{1/2})_{min}$, can be calculated using this $(k_{pE})_{max}$ in Equation 4.

(B) An example is presented in under paragraph (c)(4) of this section, to illustrate how the test data obtained in this section can be used.

(2) *Test data report.* (i) Submit the original chart, or photocopy, containing a plot of absorbance vs. wavelength plus the baseline. Spectra should include a readable wavelength scale, preferably marked at 10 nm intervals. Each spectrum should be clearly marked.

(ii) Gas phase spectra. (A) Report the pressure of the test chemical in torr (or kPa), the concentration in molecules cm$^{-3}$, and the pathlength of the sample cell in cm. Describe the method used to determine the pathlength and report the experimental data.

(B) Report the wavelength $\lambda$, the wavelength interval for each 10 nm over the region of absorption, the value of the absorbance ($A_\lambda$) for each replicate, the mean absorbance, and the mean cross section in cm$^2$ molecule$^{-1}$.

(C) Report the estimated maximum direct photolysis rate constant in days$^{-1}$ and the corresponding minimum half-life in days at 20°, 30°, 40°, and 50° north latitude for the summer and winter solstices.

(iii) Solution phase spectra. (A) Report the concentration of the test chemical in molecules cm$^{-3}$, the type of cell used (quartz or borosilicate), and the pathlength in cm. Describe the method used to determine the pathlength and report the experimental results.

(B) Report the identity of the solvent.

(C) Report the wavelength $\lambda$, the wavelength interval over the region of absorption, the value of the absorbance ($A_\lambda$) of each replicate, the mean absorbance, and the mean cross section ($\sigma'_\lambda$) in cm$^2$ molecule$^{-1}$.

(D) Report the estimated maximum direct photolysis rate constant in days$^{-1}$ and the corresponding minimum half-life in days at 20°, 30°, 40°, and 50° north latitude for the summer and winter solstices.

(iv) Report the name, structure, and purity of the test chemical.

(v) Submit a recent spectrum on appropriate reference chemicals for photometric and wavelength accuracy.

(vi) Report the name and model of the spectrophotometer used.

(vii) Report the various control settings employed with the spectrophotometer. These might include scan speed, slit width, given, etc.

(viii) Report anything unusual about the test; e.g., if the Beer-Lambert law is not obeyed at a pressure of 1 to 5 torr (0.13 to 0.65 kPa), report the pressure at which the deviation was overcome and the experimental data. If the Beer-Lambert law is not obeyed in solution at high concentrations, report the concentration at which the deviation was overcome and the experimental data.

(ix) Report any other relevant information.

(3) *Tables of solar irradiance.*

TABLE 1—$J_\lambda$ VALUES AT 20 °N. LATITUDE

| Wavelength center[1] | Summer solstice[2] | Equinox[2] | Winter solstice[2] | Wavelength center[1] | Fall or winter average[2] | Spring or summer average[2] | Wavelength center[1] |
|---|---|---|---|---|---|---|---|
| 290 | 0.0000811 | 0.00000131 | 0.000000108 | 290 | 0.000000898 | 0.0000625 | 290. |
| 300 | 0.0810 | 0.0611 | 0.0212 | 300 | 0.0350 | 0.0769 | 300. |
| 310 | 1.10 | 0.9148 | 0.489 | 310 | 0.663 | 1.05 | 310. |
| 320 | 2.74 | 2.35 | 1.52 | 320 | 1.855 | 2.82 | 320. |
| 330 | 4.82 | 4.20 | 2.90 | 330 | 3.42 | 4.63 | 330. |
| 340 | 5.27 | 4.61 | 3.28 | 340 | 3.82 | 5.06 | 340. |
| 350 | 5.94 | 5.22 | 3.77 | 350 | 4.36 | 5.71 | 350. |
| 360 | 6.22 | 5.47 | 4.01 | 360 | 4.61 | 5.96 | 360. |
| 370 | 7.76 | 6.84 | 5.06 | 370 | 5.79 | 7.48 | 370. |
| 380 | 7.80 | 6.71 | 5.02 | 380 | 5.71 | 7.31 | 380. |
| 390 | 7.77 | 6.86 | 5.19 | 390 | 5.86 | 7.48 | 390. |
| 400 | 10.6 | 9.44 | 7.17 | 400 | 8.10 | 10.2 | 400. |
| 410 | 13.5 | 12.0 | 9.17 | 410 | 10.3 | 13.0 | 410. |
| 420 | 14.1 | 12.5 | 9.65 | 420 | 10.8 | 13.6 | 420. |
| 430 | 14.3 | 12.7 | 9.85 | 430 | 11.1 | 13.7 | 430. |
| 440 | 15.8 | 14.1 | 11.0 | 440 | 12.2 | 15.2 | 440. |
| 450 | 18.2 | 16.2 | 12.7 | 450 | 14.1 | 17.5 | 450. |

TABLE 1—$J_\lambda$ VALUES AT 20 °N. LATITUDE—Continued

| Wavelength center [b] | Summer solstice [a] | Equinox [a] | Winter solstice [a] | Wavelength center [b] | Fall or winter average [a] | Spring or summer average [a] | Wavelength center [b] |
|---|---|---|---|---|---|---|---|
| 460 | 19.7 | 17.5 | 13.7 | 460 | 15.3 | 18.9 | 460. |
| 470 | 20.2 | 18.1 | 14.2 | 470 | 15.6 | 19.5 | 470. |
| 480 | 20.5 | 18.3 | 14.4 | 480 | 16.0 | 19.8 | 480. |
| 490 | 20.6 | 18.4 | 14.5 | 490 | 16.1 | 19.8 | 490. |
| 500 | 20.9 | 18.7 | 14.8 | 500 | 16.4 | 20.2 | 500. |
| 510 | 21.1 | 18.8 | 14.9 | 510 | 16.4 | 20.3 | 510. |
| 520 | 21.1 | 18.9 | 14.9 | 520 | 16.5 | 20.3 | 520. |
| 530 | 21.3 | 19.0 | 15.1 | 530 | 16.7 | 20.5 | 530. |
| 540 | 21.2 | 19.0 | 15.0 | 540 | 16.6 | 20.4 | 540. |
| 550 | 21.1 | 18.8 | 14.9 | 550 | 16.5 | 20.3 | 550. |
| 560 | 21.3 | 19.0 | 15.1 | 560 | 16.7 | 20.5 | 560. |
| 570 | 21.6 | 19.3 | 15.3 | 570 | 16.9 | 20.8 | 570. |
| 580 | 22.1 | 19.7 | 15.7 | 580 | 17.3 | 21.2 | 580. |
| 590 | 22.3 | 19.9 | 15.8 | 590 | 17.5 | 21.5 | 590. |
| 600 | 22.5 | 20.0 | 15.9 | 600 | 17.6 | 21.6 | 600. |
| 610 | 22.6 | 20.2 | 16.0 | 610 | 17.7 | 21.8 | 610. |
| 620 | 22.6 | 20.1 | 16.1 | 620 | 17.7 | 21.8 | 620. |
| 630 | 22.6 | 20.1 | 16.2 | 630 | 17.7 | 21.7 | 630. |
| 640 | 23.1 | 20.6 | 16.6 | 640 | 18.2 | 22.3 | 640. |
| 650 | 23.6 | 21.1 | 16.9 | 650 | 18.6 | 22.8 | 650. |
| 660 | 24.0 | 21.4 | 17.2 | 660 | 18.9 | 23.1 | 660. |
| 670 | 24.3 | 21.7 | 17.4 | 670 | 19.2 | 23.4 | 670. |
| 680 | 24.3 | 21.7 | 17.5 | 680 | 19.2 | 23.4 | 680. |
| 690 | 24.3 | 21.7 | 17.5 | 690 | 19.2 | 23.4 | 690. |
| 700 | 24.2 | 21.7 | 17.5 | 700 | 19.2 | 23.0 | 700. |
| 710 | 24.1 | 21.5 | 17.4 | 710 | 19.1 | 23.2 | 710. |
| 720 | 23.9 | 21.4 | 17.3 | 720 | 19.0 | 23.0 | 720. |
| 730 | 23.8 | 21.3 | 17.2 | 730 | 18.9 | 22.9 | 730. |
| 740 | 23.6 | 21.1 | 17.2 | 740 | 18.8 | 22.8 | 740. |
| 750 | 23.5 | 21.0 | 17.1 | 750 | 18.7 | 22.6 | 750. |
| 760 | 23.3 | 20.9 | 17.0 | 760 | 18.6 | 22.5 | 760. |
| 770 | 23.2 | 20.8 | 16.9 | 770 | 18.5 | 22.3 | 770. |
| 780 | 23.1 | 20.6 | 16.8 | 780 | 18.4 | 22.2 | 780. |
| 790 | 22.9 | 20.5 | 16.7 | 790 | 18.3 | 22.1 | 790. |
| 800 | 22.8 | 20.4 | 16.7 | 800 | 18.2 | 21.9 | 800. |

[a] $J_\lambda$ values are in units of $10^{19}$ photons $cm^{-2}$ $day^{-1}$.

[b] Wavelength intervals are uniformly 10 nm wide, extending from 5 nm lower than the center wavelength to 5 nm higher. Thus, the first interval centered on 290 extends from 285–295 nm.

TABLE 2—$J_\lambda$ VALUES AT 30° N. LATITUDE

| Wavelength center [b] | Summer solstice [a] | Equinox [a] | Winter solstice [a] | Wavelength center [b] | Fall or winter avg. [a] | Spring or summer avg. [a] | Wavelength center [b] |
|---|---|---|---|---|---|---|---|
| 290 | 0.0000768 | 0.00000203 | 0.000000213 | 290 | 0.000000457 | 0.0000352 | 290. |
| 300 | 0.0831 | 0.0457 | 0.00835 | 300 | 0.0208 | 0.0704 | 300. |
| 310 | 1.14 | 0.787 | 0.300 | 310 | 0.480 | 1.02 | 310. |
| 320 | 2.84 | 2.13 | 1.06 | 320 | 1.47 | 2.60 | 320. |
| 330 | 5.02 | 3.86 | 2.13 | 330 | 2.81 | 4.62 | 330. |
| 340 | 5.49 | 4.30 | 2.48 | 340 | 3.19 | 5.08 | |
| 350 | 6.28 | 4.88 | 2.89 | 350 | 3.86 | 5.74 | 350. |
| 360 | 6.49 | 5.15 | 3.10 | 360 | 3.91 | 6.02 | 360. |
| 370 | 8.09 | 6.45 | 3.95 | 370 | 4.94 | 7.51 | 370. |
| 380 | 7.93 | 6.25 | 3.95 | 380 | 4.91 | 7.37 | 380. |
| 390 | 8.12 | 6.53 | 4.12 | 390 | 5.08 | 7.55 | 390. |
| 400 | 11.1 | 8.97 | 5.73 | 400 | 7.02 | 10.4 | 400. |
| 410 | 14.1 | 11.4 | 7.37 | 410 | 8.99 | 13.2 | 410. |
| 420 | 14.7 | 12.– | 7.81 | 420 | 9.46 | 13.7 | 420. |
| 430 | 14.9 | 12.2 | 8.00 | 430 | 9.66 | 13.9 | 430. |
| 440 | 16.5 | 13.5 | 8.94 | 440 | 10.8 | 15.4 | 440. |
| 450 | 19.0 | 15.5 | 10.4 | 450 | 12.4 | 17.8 | 450. |
| 460 | 20.6 | 16.8 | 11.3 | 460 | 13.5 | 19.2 | 460. |
| 470 | 21.2 | 17.3 | 11.7 | 470 | 13.9 | 19.8 | 470. |
| 480 | 21.5 | 17.8 | 11.9 | 480 | 14.2 | 20.1 | 480. |
| 490 | 21.5 | 17.7 | 12.0 | 490 | 14.3 | 20.1 | 490. |
| 500 | 21.9 | 18.0 | 12.2 | 500 | 14.5 | 20.5 | 500. |

TABLE 2—$J_\lambda$ VALUES AT 30° N. LATITUDE—Continued

| Wavelength center [b] | Summer solstice [a] | Equinox [a] | Winter solstice [a] | Wavelength center [b] | Fall or winter avg. [a] | Spring or summer avg. [a] | Wave-length center [b] |
|---|---|---|---|---|---|---|---|
| 510 | 22.1 | 18.1 | 12.3 | 510 | 14.6 | 20.8 | 510. |
| 520 | 22.1 | 18.1 | 12.4 | 520 | 14.7 | 20.7 | 520. |
| 530 | 22.3 | 18.3 | 12.5 | 530 | 14.8 | 20.9 | 530. |
| 540 | 22.1 | 18.2 | 12.4 | 540 | 14.7 | 20.8 | 540. |
| 550 | 22.1 | 18.1 | 12.4 | 550 | 14.7 | 20.8 | 550. |
| 560 | 22.6 | 18.3 | 12.5 | 560 | 14.8 | 20.8 | 560. |
| 570 | 22.6 | 18.6 | 12.7 | 570 | 15.1 | 21.1 | 570. |
| 580 | 23.1 | 19.0 | 13.0 | 580 | 15.4 | 21.6 | 580. |
| 590 | 23.3 | 19.2 | 13.2 | 590 | 15.6 | 21.8 | 590. |
| 600 | 23.5 | 19.3 | 13.3 | 600 | 15.7 | 22.0 | 600. |
| 610 | 23.7 | 19.5 | 13.4 | 610 | 15.8 | 22.1 | 610. |
| 620 | 23.6 | 19.3 | 13.6 | 620 | 15.9 | 22.1 | 620. |
| 630 | 23.6 | 19.2 | 13.7 | 630 | 16.0 | 22.1 | 630. |
| 640 | 24.2 | 19.8 | 14.0 | 640 | 16.3 | 22.6 | 640. |
| 650 | 24.7 | 20.4 | 14.2 | 650 | 16.7 | 23.1 | 650. |
| 660 | 25.1 | 20.7 | 14.4 | 660 | 16.9 | 23.5 | 660. |
| 670 | 25.4 | 21.0 | 14.7 | 670 | 17.2 | 23.8 | 670. |
| 680 | 25.4 | 21.0 | 14.7 | 680 | 17.2 | 23.8 | 680. |
| 690 | 25.4 | 21.0 | 14.8 | 690 | 17.3 | 23.8 | 690. |
| 700 | 25.3 | 21.0 | 14.8 | 700 | 17.3 | 23.7 | 700. |
| 710 | 25.2 | 20.8 | 14.7 | 710 | 17.2 | 23.6 | 710. |
| 720 | 25.0 | 20.7 | 14.6 | 720 | 17.1 | 23.4 | 720. |
| 730 | 24.9 | 20.6 | 14.6 | 730 | 17.0 | 23.3 | 730. |
| 740 | 24.7 | 20.5 | 14.5 | 740 | 16.9 | 23.2 | 740. |
| 750 | 24.6 | 20.4 | 14.5 | 750 | 16.8 | 23.0 | 750. |
| 760 | 24.4 | 20.3 | 14.4 | 760 | 16.8 | 22.9 | 760. |
| 770 | 24.3 | 20.1 | 14.3 | 770 | 16.7 | 22.7 | 770. |
| 780 | 24.1 | 20.0 | 14.3 | 780 | 16.6 | 22.6 | 780. |
| 790 | 24.0 | 19.9 | 14.2 | 790 | 16.5 | 22.5 | 790. |
| 800 | 23.8 | 19.8 | 14.2 | 800 | 16.4 | 22.3 | 800. |

[a] $J_\lambda$ values are in units of $10^{19}$ photons cm$^{-2}$ day$^{-1}$.

[b] Wavelength intervals are uniformly 10 nm wide, extending from 5 nm lower than the center wavelength to 5 nm higher. Thus, the first interval centered on 290 extends from 285–295 nm.

TABLE 3—$J_\lambda$ VALUES AT 40° N. Latitude

| Wavelength center [b] | Summer solstice [a] | Equinox [a] | Winter solstice [a] | Wavelength center [b] | Fall or winter avg. [a] | Spring or summer avg. [a] | Wave-length center [b] |
|---|---|---|---|---|---|---|---|
| 290 | 0.0000136 | 0.000000121 | 0.000000000615 | 290 | 0.0000000814 | 0.00000349 | 290. |
| 300 | 0.0769 | 0.0293 | 0.00145 | 300 | 0.00939 | 0.0587 | 300. |
| 310 | 1.12 | 0.618 | 0.132 | 310 | 0.298 | 0.940 | 310. |
| 320 | 2.87 | 1.81 | 0.591 | 320 | 1.04 | 2.49 | 320. |
| 330 | 5.11 | 3.41 | 1.31 | 330 | 2.90 | 4.49 | 330. |
| 340 | 5.62 | 3.83 | 1.56 | 340 | 2.43 | 4.77 | 340. |
| 350 | 6.35 | 4.39 | 1.88 | 350 | 2.84 | 5.64 | 350. |
| 360 | 6.61 | 4.65 | 2.05 | 360 | 3.05 | 5.83 | 360. |
| 370 | 8.32 | 5.86 | 2.64 | 370 | 3.86 | 7.43 | 370. |
| 380 | 8.17 | 5.80 | 2.67 | 380 | 3.88 | 7.30 | 380. |
| 390 | 8.37 | 5.99 | 2.82 | 390 | 4.06 | 7.50 | 390. |
| 400 | 11.5 | 8.26 | 3.97 | 400 | 5.64 | 10.3 | 400. |
| 410 | 14.6 | 10.5 | 5.15 | 410 | 7.26 | 13.1 | 410. |
| 420 | 15.2 | 11.1 | 5.51 | 420 | 7.69 | 13.9 | 420. |
| 430 | 15.5 | 11.3 | 6.69 | 430 | 7.89 | 15.4 | 430. |
| 440 | 17.1 | 12.5 | 6.41 | 440 | 8.82 | 17.8 | 440. |
| 450 | 18.7 | 14.5 | 7.47 | 450 | 10.2 | 19.2 | 450. |
| 460 | 21.3 | 15.7 | 8.15 | 460 | 11.1 | 19.8 | 460. |
| 470 | 22.0 | 16.2 | 8.51 | 470 | 11.5 | 20.1 | 470. |
| 480 | 22.3 | 16.5 | 8.74 | 480 | 11.8 | 20.2 | 480. |
| 490 | 22.3 | 16.6 | 8.83 | 490 | 11.9 | 20.6 | 490. |
| 500 | 22.7 | 16.9 | 8.99 | 500 | 12.1 | 20.7 | 500. |
| 510 | 22.9 | 17.0 | 9.07 | 510 | 12.2 | 20.8 | 510. |
| 520 | 22.9 | 17.0 | 9.14 | 520 | 12.3 | 21.0 | 520. |
| 530 | 23.2 | 17.2 | 9.24 | 530 | 12.4 | 21.0 | 530. |
| 540 | 23.0 | 17.1 | 9.18 | 540 | 12.3 | 20.8 | 540. |
| 550 | 22.9 | 17.0 | 9.15 | 550 | 12.3 | 20.7 | 550. |

### TABLE 3—$J_\lambda$ VALUES AT 40° N. Latitude—Continued

| Wavelength center[b] | Summer solstice[a] | Equinox[a] | Winter solstice[a] | Wavelength center[b] | Fall or winter avg.[a] | Spring or summer avg.[a] | Wavelength center[b] |
|---|---|---|---|---|---|---|---|
| 560 | 23.1 | 17.2 | 9.23 | 560 | 12.4 | 20.9 | 560. |
| 570 | 23.5 | 17.4 | 9.38 | 570 | 12.6 | 21.2 | 570. |
| 580 | 24.0 | 17.8 | 9.62 | 580 | 12.9 | 21.9 | 580. |
| 590 | 24.2 | 18.0 | 9.79 | 590 | 13.1 | 21.9 | 590. |
| 600 | 24.4 | 18.2 | 9.85 | 600 | 13.2 | 22.1 | 600. |
| 610 | 24.6 | 18.3 | 9.93 | 610 | 13.2 | 22.2 | 610. |
| 620 | 24.5 | 18.3 | 10.2 | 620 | 13.4 | 22.2 | 620. |
| 630 | 24.5 | 18.3 | 10.2 | 630 | 13.5 | 22.1 | 630. |
| 640 | 25.1 | 18.8 | 10.5 | 640 | 13.8 | 22.7 | 640. |
| 650 | 25.7 | 19.2 | 10.7 | 650 | 14.1 | 23.3 | 650. |
| 660 | 26.1 | 19.5 | 10.9 | 660 | 14.3 | 23.6 | 660. |
| 670 | 26.4 | 19.8 | 11.1 | 670 | 14.5 | 24.0 | 670. |
| 680 | 26.3 | 19.9 | 11.1 | 680 | 14.6 | 24.0 | 680. |
| 690 | 26.4 | 19.9 | 11.2 | 690 | 14.6 | 24.0 | 690. |
| 700 | 26.4 | 19.9 | 11.3 | 700 | 14.7 | 24.0 | 700. |
| 710 | 26.2 | 19.8 | 11.2 | 710 | 14.6 | 23.9 | 710. |
| 720 | 26.1 | 19.7 | 11.2 | 720 | 14.6 | 23.7 | 720. |
| 730 | 25.9 | 19.6 | 11.2 | 730 | 14.5 | 23.5 | 730. |
| 740 | 25.8 | 19.5 | 11.2 | 740 | 14.5 | 23.4 | 740. |
| 750 | 25.6 | 19.4 | 11.2 | 750 | 14.4 | 23.3 | 750. |
| 760 | 25.5 | 19.3 | 11.2 | 760 | 14.4 | 23.1 | 760. |
| 770 | 25.3 | 19.2 | 11.3 | 770 | 14.3 | 23.0 | 770. |
| 780 | 25.2 | 19.1 | 11.3 | 780 | 14.3 | 22.4 | 780. |
| 790 | 25.0 | 19.0 | 11.2 | 790 | 14.2 | 22.7 | 790. |
| 800 | 24.8 | 1.89 | 11.2 | 800 | 14.1 | 22.6 | 800. |

[a] $J_\lambda$ values are in units of $10^{19}$ photons $cm^{-2}$ $day^{-1}$.

[b] Wavelength intervals are uniformly 10nm wide, extending from 5 nm lower than the center wavelength to 5 nm higher. Thus, the first interval centered on 290 extends from 285–295 nm.

### TABLE 4—$J_\lambda$ VALUES AT 50° N. Latitude

| Wavelength center[b] | Summer solstice[a] | Equinox[a] | Winter solstice[a] | Wavelength center[b] | Fall or winter avg.[a] | Spring or summer avg.[a] | Wavelength center[b] |
|---|---|---|---|---|---|---|---|
| 290 | 0.00000185 | 0.000000200 | 0.0000000112 | 290 | 0.0000000391 | 0.00000152 | 290. |
| 300 | 0.0635 | 0.0140 | 0.0000681 | 300 | 0.00296 | 0.0433 | 300. |
| 310 | 1.05 | 0.423 | 0.321 | 310 | 0.147 | 0.810 | 310. |
| 320 | 2.81 | 1.41 | 0.214 | 320 | 0.610 | 2.28 | 320. |
| 330 | 5.10 | 2.78 | 0.555 | 330 | 1.33 | 4.23 | 330. |
| 340 | 5.64 | 3.19 | 0.711 | 340 | 1.59 | 4.73 | 340. |
| 350 | 6.41 | 3.70 | 0.864 | 350 | 1.98 | 5.40 | 360. |
| 360 | 6.70 | 3.96 | 0.953 | 360 | 2.04 | 5.71 | 350. |
| 370 | 8.46 | 5.03 | 1.25 | 370 | 2.63 | 7.18 | 370. |
| 380 | 8.32 | 5.01 | 1.28 | 380 | 2.66 | 7.09 | 380. |
| 390 | 8.56 | 5.21 | 1.37 | 390 | 2.80 | 7.31 | 390. |
| 400 | 11.8 | 7.22 | 1.95 | 400 | 3.93 | 10.1 | 400. |
| 410 | 15.0 | 9.27 | 2.57 | 410 | 5.09 | 12.8 | 410. |
| 420 | 15.7 | 9.79 | 2.79 | 420 | 5.45 | 13.5 | 420. |
| 430 | 15.9 | 10.0 | 2.92 | 430 | 5.62 | 13.7 | 430. |
| 440 | 17.8 | 11.2 | 3.33 | 440 | 6.33 | 15.2 | 440. |
| 450 | 20.3 | 12.9 | 3.92 | 450 | 7.37 | 17.6 | 450. |
| 460 | 22.0 | 14.0 | 4.31 | 460 | 8.06 | 19.0 | 460. |
| 470 | 22.7 | 14.5 | 4.54 | 470 | 8.40 | 19.7 | 470. |
| 480 | 23.1 | 14.8 | 4.70 | 480 | 8.62 | 20.0 | 480. |
| 490 | 23.1 | 15.0 | 4.78 | 490 | 8.72 | 20.1 | 490. |
| 500 | 23.5 | 15.2 | 4.88 | 500 | 8.87 | 20.4 | 500. |
| 510 | 23.7 | 15.3 | 4.94 | 510 | 9.00 | 20.6 | 510. |
| 520 | 23.8 | 15.4 | 4.98 | 520 | 9.03 | 20.6 | 520. |
| 530 | 24.0 | 15.6 | 5.05 | 530 | 9.12 | 20.8 | 530. |
| 540 | 23.8 | 15.5 | 5.02 | 540 | 9.07 | 20.7 | 540. |
| 550 | 23.7 | 15.4 | 5.01 | 550 | 9.05 | 20.6 | 550. |
| 560 | 24.0 | 15.5 | 5.04 | 560 | 9.11 | 20.8 | 560. |
| 570 | 24.3 | 15.8 | 5.11 | 570 | 9.28 | 21.1 | 570. |
| 580 | 24.8 | 16.1 | 5.27 | 580 | 9.50 | 21.6 | 580. |
| 590 | 25.1 | 16.4 | 5.38 | 590 | 9.66 | 21.8 | 590. |
| 600 | 25.3 | 16.5 | 5.42 | 600 | 9.73 | 22.0 | 600. |

TABLE 4—$J_\lambda$ VALUES AT 50° N. Latitude—Continued

| Wavelength center [b] | Summer solstice [a] | Equinox [a] | Winter solstice [a] | Wavelength center [b] | Fall or winter avg. [a] | Spring or summer avg. [a] | Wavelength center [b] |
|---|---|---|---|---|---|---|---|
| 610 | 25.5 | 16.6 | 5.47 | 610 | 9.80 | 22.1 | 610. |
| 620 | 25.4 | 16.8 | 5.61 | 620 | 9.96 | 22.1 | 620. |
| 630 | 25.3 | 17.0 | 5.77 | 630 | 10.1 | 22.1 | 630. |
| 640 | 26.0 | 17.3 | 5.93 | 640 | 10.4 | 22.7 | 640. |
| 650 | 26.7 | 17.6 | 6.10 | 650 | 10.6 | 23.3 | 650. |
| 660 | 27.1 | 17.8 | 6.24 | 660 | 10.8 | 23.6 | 660. |
| 670 | 27.5 | 18.1 | 6.39 | 670 | 11.0 | 24.0 | 670. |
| 680 | 27.5 | 18.2 | 6.47 | 680 | 11.0 | 24.0 | 680. |
| 690 | 27.5 | 18.2 | 6.56 | 690 | 11.1 | 24.0 | 690. |
| 700 | 27.5 | 18.2 | 6.64 | 700 | 11.2 | 24.0 | 700. |
| 710 | 27.3 | 18.1 | 6.67 | 710 | 11.2 | 29.9 | 710. |
| 720 | 27.2 | 18.1 | 6.72 | 720 | 11.2 | 23.8 | 720. |
| 730 | 27.0 | 18.0 | 6.75 | 730 | 11.2 | 23.6 | 730. |
| 740 | 26.9 | 17.9 | 6.78 | 740 | 11.2 | 23.5 | 740. |
| 750 | 26.7 | 17.8 | 6.82 | 750 | 11.1 | 23.4 | 750. |
| 760 | 26.6 | 17.8 | 6.82 | 760 | 11.1 | 23.3 | 760. |
| 770 | 26.4 | 17.7 | 6.82 | 770 | 11.1 | 23.1 | 770. |
| 780 | 26.3 | 17.6 | 6.82 | 780 | 11.1 | 23.0 | 780. |
| 790 | 2.61 | 17.5 | 6.80 | 790 | 11.0 | 22.9 | 790. |
| 800 | 26.0 | 17.4 | 6.80 | 800 | 11.0 | 22.8 | 800. |

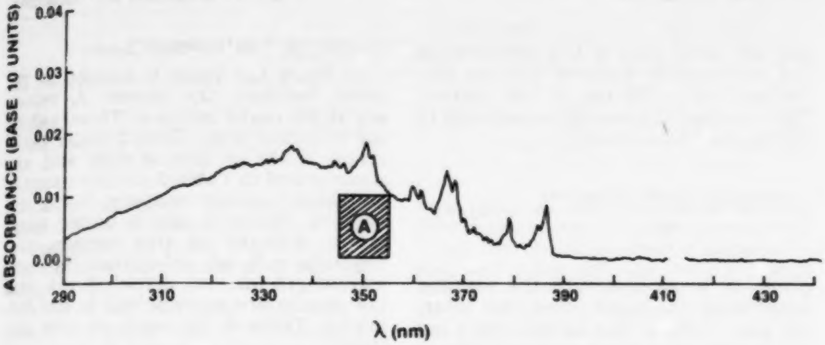
[a] $J_\lambda$ values are in units of $10^{19}$ photons $cm^{-2}$ $day^{-1}$.

[b] Wavelength intervals are uniformly 10 nm wide, extending from 5 nm lower than the center wavelength to 5 nm higher. Thus, the first interval centered on 290 extends from 285–295 nm.

(4) *Example of application of methodology.* (i) Consider a chemical plant located in Freeport, Texas, which produces acrolein $[CH_2=CHCHO]$ continuously every day of the year. Despite the fact that all acrolein wastes, including vented vapors, are treated in a waste-treatment plant, some acrolein escapes into the atmosphere. The chemical plant is located at 29° north latitude. Estimate the maximum sunlight direct photolysis rate constant and the corresponding minimum half-life in the atmosphere in the vicinity of the plant for the winter and summer season solstices under clear sky conditions.

(ii) The vapor phase spectrum of acrolein was obtained by the procedure outlined in this test method and is depicted in the following Figure 3:

FIGURE 3—GAS PHASE ABSORPTION SPECTRUM OF ACROLEIN



The spectral data were taken from the work of Pitts et al. (1981) under paragraph (d)(2) of this section. The pathlength of the sample gas absorption cell was measured according to the recommended procedure and was found to be 9.98 cm. The gas absorption cell contained $6.52 \times 10^{16}$ molecules $cm^{-3}$ of acrolein. A compensating polar planimeter was used to integrate each 10 nm interval throughout the region of absorption from 285 nm to 425 nm in both the sample and blank spectra. Based on triplicate measurements, one square, corresponding to 0.001 absorbance units (A), was found to be 0.148 vernier units (v.u.). The mean absorbance ($A_\lambda$) was obtained from these spectra and the mean cross section ($\sigma'_\lambda$) was obtained using Equation 7 under paragraph (c)(1)(i)(A) of this section for each wavelength interval, centered at λ. All the results are summarized in the following Table 5:

TABLE 5—ABSORBANCE AND CROSS SECTION FOR ACROLEIN VAPOR[1]

| Wavelength λ (nm) | Wavelength interval (nm) | Mean absorbance [Aλ] | Mean cross section $\sigma'_\lambda$] (cm² molecule⁻¹) |
|---|---|---|---|
| 290 | 285–295 | 0.0037 | $5.69 \times 10^{-21}$ |
| 300 | 295–305 | 0.0066 | $1.01 \times 10^{-20}$ |
| 310 | 305–315 | 0.0104 | $1.60 \times 10^{-20}$ |
| 320 | 315–325 | 0.0137 | $2.11 \times 10^{-20}$ |
| 330 | 325–335 | 0.0156 | $2.40 \times 10^{-20}$ |
| 340 | 335–345 | 0.0156 | $2.40 \times 10^{-20}$ |
| 350 | 345–355 | 0.0151 | $2.32 \times 10^{-20}$ |
| 360 | 355–365 | 0.0096 | $1.48 \times 10^{-20}$ |
| 370 | 365–375 | 0.0073 | $1.12 \times 10^{-20}$ |
| 380 | 375–385 | 0.0031 | $4.76 \times 10^{-21}$ |
| 390 | 385–395 | 0.0016 | $2.46 \times 10^{-21}$ |
| 400 | 395–405 | 0.0004 | $6.15 \times 10^{-22}$ |
| 410 | 405–415 | 0.0003 | $4.61 \times 10^{-22}$ |
| 420 | 415–425 | 0.0000 | 0.00 |

[1] $6.52 \times 10^{16}$ molecules $cm^{-3}$ in a 9.98 cm gas absorption cell.

(iii) A sample calculation is given for the wavelength λ=305 nm centered over the wavelength interval 345 to 355 nm. For convenience, the area A, corresponding to 100 squares was blocked off in this absorption area and was not integrated with the planimeter. The average vernier reading of the remaining absorption area was 7.2 v.u. Hence,

$$\frac{7.2 \text{ v.u}}{0.148 \text{ v.u./square}} = 49 \text{ squares,}$$

and the total area in the spectrum in the wavelength interval 345 to 355, centered at $\lambda=350$ nm, is 149 squares. This number of squares corresponds to 0.0149 absorbance units:

$$\frac{(149 \text{ squares})(0.001 \text{ A/square})}{10} = 0.0149 \text{ A.}$$

From the blank spectrum, the baseline absorbance ($A_\lambda$ blank) over this interval was −0.0001. The sample trace lay at −0.0001 absorbance units relative to a zero point at 450 nm. The observed sample absorbance is then equal to 0.0150 (0.0149+0.0001). The absolute corrected absorbance for the sample is given by

$A^{\text{corr.}}{}_{\lambda\text{sample}} = A^{\text{obs.}}{}_{\lambda\text{sample}} - A_{\lambda \text{ blank}}$

$A^{\text{corr.}}{}_{\lambda\text{sample}} = 0.0150 - (-0.001) = 0.0151$ A.

(iv) Using Equation 7 under paragraph (c)(1)(i)(A) of this section and the values for the corrected sample absorbance, l, and C, the mean cross section for the wavelength $\lambda=350$ nm, centered over the wavelength interval 345–355 nm, is

$$\sigma'_\lambda = \frac{0.0151}{6.52\times10^{16} \text{ molecules cm}^{-3}(9.98\text{cm})}$$

$\sigma'_\lambda = 2.3\times10^{-20}$ cm$^2$ molecule$^{-1}$.

(v) Since the plant is located at 29° north latitude, the closest $J_\lambda$ values are at 30° north latitude. These values are obtained from Table 2 under paragraph (c)(3) of this section and are summarized in Table 6 for the summer and winter season solstices. Using the data in Tables 5 and 6 under paragraph (c)(4)(ii) of this section, the products $\sigma_\lambda J_\lambda$ are calculated for each wavelength interval, centered at λ, and the results are summarized in the following Table 6 for each of the solstices:

| Summer | Winter |
|---|---|
| $(k_{pE})_{max} = 16.9$ days$^{-1}$<br>$(t_{1/2})_{min} = 0.041$ days | $(k_{pE})_{max} = 7.60$ days$^{-1}$<br>$(t_{1/2})_{min} = 0.091$ days. |

The terms $\Sigma\sigma_\lambda J_\lambda$ are also summarized for each solstice at the bottom of Table 6. Using these data in Equations 3 and 4 yields:

| Summer | Winter |
|---|---|
| $(k_{pE})_{max} = 16.9$ days$^{-1}$<br>$(t_{1/2})_{min} = 0.041$ days | $(k_{pE})_{max} = 7.60$ days$^{-1}$<br>$(t_{1/2})_{min} = 0.091$ days. |

Thus, acrolein transforms rapidly under clear sky conditions in the vicinity of the plant at Freeport, Texas on the summer and winter season solstices.

TABLE 6—CALCULATION OF $(K_{pE})_{max}$ FOR ACROLEIN VAPOR; RATE AT 30 °N ON WINTER AND SUMMER SOLSTICES

| Wavelength λ (nm) | Wavelength interval (nm) | Summer solstice | | Winter solstice | |
|---|---|---|---|---|---|
| | | $J_\lambda$ photons (cm$^{-2}$ day$^{-1}$) | $\sigma'_\lambda J_\lambda$ (day$^{-1}$) | $J_\lambda$ photons (cm$^{-2}$ day$^{-1}$) | $\sigma'_\lambda J_\lambda$ (day$^{-1}$) |
| 290 | 285–295 | $1.0\times10^{15}$ | 0.000 | $2.1\times10^{13}$ | 0.000 |
| 300 | 295–305 | $8.31\times10^{17}$ | 0.008 | $8.35\times10^{16}$ | 0.001 |
| 310 | 305–315 | $1.14\times10^{19}$ | 0.182 | $3.00\times10^{18}$ | 0.046 |
| 320 | 315–325 | $2.64\times10^{19}$ | 0.599 | $1.06\times10^{19}$ | 0.224 |
| 330 | 325–335 | $5.02\times10^{19}$ | 1.205 | $2.13\times10^{19}$ | 0.511 |
| 340 | 335–345 | $5.49\times10^{19}$ | 1.318 | $2.48\times10^{19}$ | 0.595 |
| 350 | 345–355 | $6.28\times10^{19}$ | 1.457 | $2.89\times10^{19}$ | 0.671 |
| 360 | 355–365 | $6.49\times10^{19}$ | 0.961 | $3.10\times10^{19}$ | 0.459 |
| 370 | 365–375 | $8.09\times10^{19}$ | 0.906 | $3.95\times10^{19}$ | 0.442 |
| 380 | 375–385 | $7.93\times10^{19}$ | 0.378 | $3.95\times10^{19}$ | 0.188 |
| 390 | 385–395 | $8.12\times10^{19}$ | 0.200 | $4.12\times10^{19}$ | 0.101 |
| 400 | 395–405 | $1.11\times10^{20}$ | 0.068 | $5.73\times10^{19}$ | 0.035 |

TABLE 6—CALCULATION OF $(K_{pE})_{max}$ FOR ACROLEIN VAPOR; RATE AT 30 °N ON WINTER AND SUMMER SOLSTICES—Continued

| Wavelength λ (nm) | Wavelength interval (nm) | Summer solstice | | Winter solstice | |
|---|---|---|---|---|---|
| | | $J_\lambda$ photons ($cm^{-2}$ $day^{-1}$) | $\sigma'_\lambda J_\lambda$ ($day^{-1}$) | $J_\lambda$ photons ($cm^{-2}$ $day^{-1}$) | $\sigma'_\lambda J_\lambda$ ($day^{-1}$) |
| 410 | 405–415 | $1.41\times10^{20}$ | 0.065 | $7.37\times10^{19}$ | 0.034 |
| 420 | 415–425 | $1.47\times10^{20}$ | 0.000 | $7.81\times10^{19}$ | 0.000 |
| | | | $\Sigma\sigma'_\lambda J_\lambda=7.34_7$ | | $\Sigma\sigma'_\lambda J_\lambda=3.30_4$ |

(5) *Operation of the gas handling system.* The following procedure briefly describes the recommended typical and detailed operation of a gas handling system.

(i) Close all stopcocks and turn on the rotary pump (A). Open stopcock 4 and place a Dewar containing liquid nitrogen around trap (D). Measure the pressure with the thermocouple gauge $H_1$. When the pressure is less than 0.1 torr (0.013 kPa) open stopcocks 5 and 10, pump out this portion of the manifold, and measure the pressure with the thermocouple gauge $H_2$. When the pressure falls below $10^{-2}$ torr ($1.3\times10^{-3}$ kPa), open stopcock 7 and evacuate F containing activated Linde Molecular Sieve 4A or an equivalent grade. Heat F to approximately 150 °C for 1 to 2 hours under vacuum until the pressure falls to less than $10^{-2}$ torr ($1.3\times10^{-3}$ kPa) as measured on thermocouple gauge $H_2$. Open stopcocks 6, 9, and 11 and pump until $H_2$ falls below $10^{-2}$ torr ($1.3\times10^{-3}$ kPa).

(ii) Turn on the diffusion pump (C) and when this pump has reached operating temperature, open stopcocks 2 and 3 and close stopcock 4. Pump on the manifold until the pressure is $<10^{-5}$ torr ($1.3\times10^{-6}$ kPa) as measured by the ionization gauge (I) and zero on the capacitance manometer (G). It should be noted that the ionization gauge (I) should only be used when $H_2$ indicates a pressure less than $10^{-2}$ torr ($1.3\times10^{-3}$ kPa).

(iii) It is good practice, after the gas phase spectrum has been obtained, to evacuate the gas absorption cell (K) and the trap (F) prior to shutting down the gas handling system. The gas handling system can be shut down by the following procedure: (A) closing stopcocks 5 to 11; (B) switching off the diffusion pump; (C) closing stopcocks 2 and 3 and opening 4, after the diffusion pump is cool; (D) removing the Dewar from trap (D) and allowing it to warm up; (E) then closing stopcock 4 and switching off the rotary pump; and (F) opening stopcock 1 to admit air to the rotary pump, thus preventing suck-back of the rotary pump oil. With this procedure, the vacuum manifold, the trap (D), and the diffusion pump are left under vacuum. The method of cleaning the liquid reservoir (J) is left to the discretion of the tester. However, as a final step it should be cleaned with reagent grade methanol or dichloromethane as solvent and dried. It is then ready for use. In operating a vacuum system with the diffusion pump working, do not expose the diffusion pump to pressures >0.1 torr of air ($1.3\times10^{-2}$ kPa) to avoid the degradation of the pump oil.

(d) *References.* For additional background information on this test guideline, the following references should be consulted:

(1) Mill, T., Mabey, W.R., Bomberger, D.C., Chou, T-W., Hendry, D.G., and Smith, J.H. *Laboratory Protocols for Evaluating the Fate of Organic Chemicals in Air and Water.* Chapter 5. EPA 600/3-82-022 (1982).

(2) Pitts, J.N., Jr., Winer, A.M., Fitz, D.R., Knudsen, A.K., and Atkinson, R. *Experimental Protocol for Determining Absorption Cross Sections of Organic Chemicals,* EPA Report No. 600/3-81-051 (1981).

(3) U.S. Environmental Protection Agency. Mill, T., Davenport, J.E., Winterle, J.S., Maby, W.R., Drossman, H., Tse, D., and Lin, A. "Section 5. Photolysis in Air," by J.E., Davenport, *Toxic Substances Process Generation and Protocol Development.* Work Assignment 12, Draft final report. (Athens, Georgia, and Washington, DC, 1984).

[50 FR 39252, Sept. 27, 1985]

# PART 797—ENVIRONMENTAL EFFECTS TESTING GUIDELINES

Subpart A—[Reserved]

Subpart B—Aquatic Guidelines

Sec.
797.1050 Algal acute toxicity test.
797.1060 Freshwater algae acute toxicity test.
797.1075 Freshwater and marine algae acute toxicity test.
797.1160 Lemna acute toxicity test.
797.1300 Daphnid acute toxicity test.
797.1330 Daphnid chronic toxicity test.
797.1350 Daphnid chronic toxicity test.
797.1400 Fish acute toxicity test.
797.1440 Fish acute toxicity test.
797.1520 Fish bioconcentration test.
797.1560 Fish bioconcentration test.
797.1600 Fish early life stage toxicity test.
797.1800 Oyster acute toxicity test.
797.1830 Oyster bioconcentration test.
797.1930 Mysid shrimp acute toxicity test.
797.1950 Mysid shrimp chronic toxicity test.
797.1970 Penaeid shrimp acute toxicity test.

Subpart C—Terrestrial Guidelines

797.2050 Avian dietary toxicity test.
797.2130 Bobwhite reproduction test.
797.2150 Mallard reproduction test.
797.2175 Avian acute oral toxicity test.
797.2750 Seed germination/root elongation toxicity test.
797.2800 Early seedling growth toxicity test.
797.2850 Plant uptake and translocation test.

AUTHORITY: 15 U.S.C. 2603.

SOURCE: 50 FR 39321, Sept. 27, 1985, unless otherwise noted.

## Subpart A—[Reserved]

## Subpart B—Aquatic Guidelines

### § 797.1050 Algal acute toxicity test.

(a) *Purpose.* The guideline in this section is intended for use in developing data on the acute toxicity of chemical substances and mixtures ("chemicals") subject to environmental effects test regulations under the Toxic Substances Control Act (TSCA) (Pub. L. 94-469, 90 Stat. 2003, 15 U.S.C. 2601 et seq.). This guideline prescribes test procedures and conditions using freshwater and marine algae to develop data on the phytotoxicity of chemicals. The United States Environmental Protection Agency (U.S. EPA) will use data from these tests in assessing the hazard of a chemical to the environment.

(b) *Definitions.* The definitions in section 3 of the Toxic Substances Control Act (TSCA) and the definitions in Part 792—Good Laboratory Practice Standards of this chapter apply to this test guideline. The following definitions also apply to this guideline:

(1) "Algicidal" means having the property of killing algae.

(2) "Algistatic" means having the property of inhibiting algal growth.

(3) "ECx" means the experimentally derived chemical concentration that is calculated to effect X percent of the test criterion.

(4) "Growth" means a relative measure of the viability of an algal population based on the number and/or weight of algal cells per volume of nutrient medium or test solution in a specified period of time.

(5) "Static system" means a test container in which the test solution is not renewed during the period of the test.

(c) *Test procedures*—(1) *Summary of the test.* (i) In preparation for the test, fill test containers with appropriate volumes of nutrient medium and/or test solution. Start the test by introducing algae into the test and control containers in the growth chambers. Environmental conditions within the growth chambers are established at predetermined limits.

(ii) At the end of 96 hours enumerate the algal cells in all containers to determine inhibition or stimulation of growth in test containers compared to controls. Use data to define the concentration-response curve, and calculate the $EC_{10}$, $EC_{50}$, and $EC_{90}$ values.

(2) [Reserved]

(3) *Range-finding test.* (i) A range-finding test should be conducted to determine:

(A) If definitive testing is necessary.

(B) Test chemical concentrations for the definitive test.

(ii) Algae are exposed to a widely spaced (e.g., log interval) chemical concentration series. The lowest value in the series, exclusive of controls, should be at the chemical's detection limit. The upper value, for water soluble compounds, should be the saturation concentration. No replicates are required; and nominal concentrations of the chemical are acceptable unless definitive testing is not required

(iii) The test is performed once for each of the recommended algal species or selected alternates. Test chambers should contain equal volumes of test solution and approximately $1 \times 10^4$ *Selenastrum* cells/ml or $7.7 \times 10^4$ *Skeletonema* cells/ml of test solution. The algae should be exposed to each concentration of test chemical for up to 96 hours. The exposure period may be shortened if data suitable for the purposes of the range-finding test can be obtained in less time.

(iv) Definitive testing is not necessary if the highest chemical concentration tested (water saturation concentration or 1000 mg/l) results in less than a 50 percent reduction in growth or if the lowest concentration tested (analytical detection limit) results in greater than a 50 percent reduction in growth.

(4) *Definitive test.* (i) The purpose of the definitive test is to determine the concentration response curves, the $EC_{10}$'s, $EC_{50}$'s, and $EC_{90}$'s for algal growth for each species tested, with a minimum amount of testing beyond the range-finding test.

(ii) Algae should be exposed to five or more concentrations of the test chemical in a geometric series in which the ratio is between 1.5 and 2.0 (e.g., 2, 4, 8, 16, 32, and 64 mg/l). Algae shall be placed in a minimum of three replicate test containers for each concentration of test chemical and control. More than three replicates may be required to provide sufficient quantities of test solution for determination of test substance concentration at the end of the test. Each test chamber should contain equal volumes of test solution and approximately $1 \times 10^4$ *Selenastrum* cells/ml or $7.7 \times 10^4$ *Skeletonema* cells/ml of test solution. The chemical concentrations should result in greater than 90 percent of algal growth being inhibited or stimulated at the highest concentrations of test substance compared to controls.

(iii) Every test shall include a control consisting of the same nutrient medium, conditions, procedures, and algae from the same culture, except that none of the test substance is added. If a carrier is present in any of the test chambers, a separate carrier control is required.

(iv) The test begins when algae from 5- to 10-day-old stock cultures are placed in the test chambers containing test solutions having the appropriate concentrations of the test substance. Algal growth in controls should reach the logarithmic growth phase by 96 hours. If logarithmic growth cannot be demonstrated, the test shall be repeated. At the end of 24, 48, 72, and 96 hours the algal growth response (number or weight of algal cells/ml) in all test containers and controls shall be determined by an indirect (spectrophotometry, electronic cell counters, dry weight, etc.) or a direct (actual microscopic cell count) method. Indirect methods shall be calibrated by a direct microscopic count. The percentage inhibition or stimulation of growth for each concentration, $EC_{10}$, $EC_{50}$, $EC_{90}$ and the concentration-response curves are determined from these counts.

(v) At the end of the definitive test, the following additional analyses of algal growth response shall be performed:

(A) Determine whether the altered growth response between controls and test algae was due to a change in relative cell numbers, cell sizes or both. Also note any unusual cell shapes, color differences, flocculations, adherence of algae to test containers, or aggregation of algal cells.

(B) In test concentrations where growth is maximally inhibited, algistatic effects may be differentiated from algicidal effects by the following two methods for *Skeletonema* and by the second method for *Selenastrum*.

(*1*) Add 0.5 ml of a 0.1 percent solution (weight/volume) of Evans blue stain to a 1 milliliter aliquot of algae from a control container and to a 1 milliliter aliquot of algae from the test container having the lowest concentration of test chemical which completely inhibited algal growth (if algal growth was not completely inhibited, select an aliquot of algae for staining from the test container having the highest concentration of test chemical which inhibited algal growth). Wait 10 to 30 minutes, examine microscopically, and determine the percent of the cells which stain blue (indicating cell mortality). A staining control shall be performed concurrently using heat-killed or formaldehyde-preserved algal cells; 100 percent of these cells shall stain blue.

(*2*) Remove 0.5 ml aliquots of test solution containing growth-inhibited algae from each replicate test container having the concentration of test substance evaluated in paragraph (c)(4)(v)(B)(*1*) of this section. Combine these aliquots into a new test container and add a sufficient volume of fresh nutrient medium to dilute the test chemical to a concentration which does not affect growth. Incubate this subculture under the environmental conditions used in the definitive test for a period of up to 9 days, and observe for algal growth to determine if the algistatic effect noted after the 96-hour test is reversible. This subculture test may be discontinued as soon as growth occurs.

(5) [Reserved]

(6) *Analytical measurements*—(i) *Chemical.* (A) Glass distilled or deionized water shall be used in the preparation of the nutrient medium. The pH of the test solution shall be measured in the control and test containers at the beginning and at the end of the definitive test. The concentration of test chemical in the test containers shall be determined at the beginning and end of the definitive test by standard analytical methods which have been validated prior to the test. An analytical method is unacceptable if likely degradation products of the chemical, such as hydrolysis and oxidation products, give positive or negative interference.

(B) At the end of the test and after aliquots have been removed for algal growth-response determinations, microscopic examination, mortal staining, or subculturing, the replicate test containers for each chemical concentration may be pooled into one sample. An aliquot of the pooled sample may then be taken and the concentration of test chemical determined. In addition, the concentration of test chemical associated with the algae alone should be determined. Separate and concentrate the algal cells from the test solution by centrifuging or filtering the remaining pooled sample and measure the test substance concentration in the algal-cell concentrate.

(ii) *Numerical.* Algal growth response (as percent of inhibition or stimulation in the test solutions compared to the controls) is calculated at the end of the test. Mean and standard deviation should be calculated and plotted for each treatment and control. Appropriate statistical analyses should provide a goodness-of-fit determination for the concentration response curves. The concentration response curves are plotted using the mean measured test solution concentrations obtained at the end of the test.

(d) *Test conditions*—(1) *Test species.* Species of algae recommended as test organisms for this test are the freshwater green alga, *Selenastrum capricornutum*, and the marine diatom, *Skeletonema costatum*. Algae to be used in acute toxicity tests may be initially obtained from commercial sources and subsequently cultured using sterile technique. Toxicity testing shall not be performed until algal cultures are shown to be actively growing (i.e., capable of logarithmic growth within the test period) in at least 2 subcultures lasting 7 days each prior to the start of the definitive test. All algae used for a particular test shall be from the same source and the same stock culture. Test algae shall not have been used in a previous test, either in a treatment or a control.

(2) *Facilities*—(i) *General.* (A) Facilities needed to perform this test include: a growth chamber or a controlled environment room that can hold the test containers and will maintain the air temperature, lighting intensity and photoperiod specified in this test guideline; apparatus for culturing and enumerating algae; a source of distilled and/or deionized water; and apparatus for carrying out analyses of the test chemical.

(B) Disposal facilities should be adequate to accommodate spent glassware, algae and test solutions at the end of the test and any bench covering, lab clothing, or other contaminated materials.

(ii) *Test containers.* Erlenmeyer flasks should be used for test containers. The flasks may be of any volume between 125 and 500 ml as long as the same size is used throughout a test and the test solution volume does not exceed 50 percent of the flask volume.

(iii) *Cleaning and sterilization.* New test containers may contain substances which inhibit growth of algae. They shall therefore be cleaned thoroughly and used several times to culture algae before being used in toxicity testing. All glassware used in algal culturing or testing shall be cleaned and sterilized prior to use according to standard good laboratory practices.

(iv) *Conditioning.* Test containers should be conditioned by a rinse with the appropriate test solutions prior to the start of the test. Decant and add fresh test solutions after an appropriate conditioning period for the test chemical.

(v) *Nutrient medium.* (A) Formulation and sterilization of nutrient medium used for algal culture and preparation of test solutions should conform to those currently recommended by the U.S. EPA for freshwater and marine algal bioassays. No chelating agents are to be included in the nutrient medium used for test solution preparation. Nutrient medium should be freshly prepared for algal testing and may be dispensed in appropriate volumes in test containers and sterilized by autoclaving or filtration. The pH of the nutrient medium shall be 7.5 (±0.1) for *Selenastrum* and 8.1 (±0.1) for *Skeletonema* at the start of the test and may be adjusted prior to test chemical addition with 0.1N NaOH or HC1.

(B) Dilution water used for preparation of nutrient medium and test solutions should be filtered, deionized or glass distilled. Saltwater for marine algal nutrient medium and test solutions should be prepared by adding a commercial, synthetic, sea salt formulation or a modified synthetic seawater formulation to distilled/deionized water to a concentration of 30 parts per thousand.

(vi) *Carriers.* Nutrient medium shall be used in making stock solutions of the test chemical. If a carrier other than nutrient medium is absolutely necessary to dissolve the chemical, the volume used shall not exceed the minimum volume necessary to dissolve or suspend the chemical in the test solution.

(3) *Test parameters.* (i) The test temperature shall be 24 °C for *Selenastrum* and 20 °C for *Skeletonema.* Excursions from the test temperature shall be no greater than ±2 °C. Temperature should be recorded hourly during the test.

(ii) Test chambers containing *Selenastrum* shall be illuminated continuously and those containing *Skeletonema* shall be provided a 14-hour light and 10-hour dark photoperiod with a 30 minute transition period under fluorescent lamps providing 300 ± 25 uEin/m$^2$ sec (approximately 400 ft-c) measured adjacent to the test chambers at the level of test solution.

(iii) Stock algal cultures should be shaken twice daily by hand. Test containers shall be placed on a rotary shaking apparatus and oscillated at approximately 100 cycles/minute for *Selenastrum* and at approximately 60 cycles/minute for *Skeletonema* during the test. The rate of oscillation should be determined at least once daily during testing.

(iv) The pH of nutrient medium in which algae are subcultured shall be 7.5 (±0.1) for *Selenastrum* and 8.1 (±0.1) for *Skeletonema,* and is not adjusted after the addition of the algae. The pH of all test solutions shall be measured at the beginning and end of the test.

(v) Light intensity shall be monitored at least daily during the test at the level of the test solution.

(e) *Reporting.* The sponsor shall submit to the EPA all data developed by the test that are suggestive or predictive of acute phytotoxicity. In addition to the general reporting requirements prescribed in Part 792—*Good Laboratory Practice Standards of this Chapter,* the following shall be reported:

(1) Detailed information about the test organisms, including the scientific name, method of verification, and source.

(2) A description of the test chambers and containers, the volumes of solution in the containers, the way the

test was begun (e.g., conditioning, test substance additions, etc.), the number of replicates, the temperature, the lighting, and method of incubation, oscillation rates, and type of apparatus.

(3) The concentration of the test chemical in the control and in each treatment at the end of the test and the pH of the solutions.

(4) The number of algal cells per milliliter in each treatment and control and the method used to derive these values at the beginning, 24, 48, and 72 hours, and end of the test; the percentage of inhibition or stimulation of growth relative to controls; and other adverse effect in the control and in each treatment.

(5) The 96-hour $EC_{10}$, $EC_{50}$, and $EC_{90}$ values, and when sufficient data have been generated, the 24, 48, and 72 hour $LC_{50}$'s and 95 percent confidence limits, the methods used to derive these values, the data used to define the shape of the concentration-response curve and the goodness-of-fit determination.

(6) Methods and data records of all chemical analyses of water quality and test substance concentrations, including method validations and reagent blanks.

(7) The results of any optional analyses such as: Microscopic appearance of algae, size or color changes, percent mortality of cells and the fate of subcultured cells, the concentration of test substance associated with algae and test solution supernate or filtrate.

(8) If the range-finding test showed that the highest concentration of the chemical tested (not less than 1000 mg/l or saturation concentration) had no effect on the algae, report the results and concentration and a statement that the chemical is of minimum phytotoxic concern.

(9) If the range-finding test showed greater than a 50 percent inhibition of algal growth at a test concentration below the analytical detection limit, report the results, concentration, and a statement that the chemical is phytotoxic below the analytical detection limit.

[50 FR 39321, Sept. 27, 1985, as amended at 52 FR 19058, May 20, 1987]

## § 797.1060 Freshwater algae acute toxicity test.

(a) *Purpose.* (1) A unicellular green alga is used as a model system initially to estimate the concentration of a chemical which could affect the primary production of plants. Because regeneration times for unicellular algal species are measured in hours, this relatively short test can assess effects over several generations. Results allow the assessment of effects on initial organisms from short term exposures and give an indication of the effect on algal populations.

(2) Many different protocols for algal tests are available. This growth test is easy to perform and gives reproducible results with the recommended species. This Test Guideline can be adapted for other algal species. If such an adaptation is used, a description of the method should be provided with the test report.

(b) *Definitions.* The following definitions apply to this guideline:

(1) "EC-X" means the experimentally derived chemical concentration that is calculated to effect X percent of the test criterion.

(2) "Growth rate" means an increase in biomass or cell numbers of algae per unit time.

(3) "Inhibition" means any decrease in the growth rate of the test algae compared to the control algae.

(4) "Limited water-soluble substances" means chemicals which are soluble in water at less than 1,000 mg/l.

(5) "Readily water-soluble substances" means chemicals which are soluble in water at a concentration equal to or greater than 1,000 mg/l.

(c) *Test procedures*—(1) *Summary of the test.* (i) The procedures for the preparation of the algal suspension, the stock solution of the test chemical, and the test media, are dependent on the solubility of the chemical and modifications in the testing procedure may be necessary due to the chemical's solubility in water. For chemicals with low solubility under test conditions, it may not be possible to quantitatively determine the $EC_{50}$.

(ii) For purposes of the test, algae are grown in Erlenmeyer flasks in an environmentally controlled growth chamber. The test is started when 50 ml of algal suspension ($1 \times 10^4$ or $2 \times 10^4$ cells/ml) and 50 ml of the appropriate test chemical dilutions are placed in the flasks. Algal growth is measured at 24 hour intervals for at least 96 hours. A Coulter Counter, counting chamber, or other appropriate instruments may be used to determine cell density. The data are used to define the concentration-response curve and the time-growth curve, and to calculate the $EC_{50}$ and the no-effect level for ($EC_0$) for the chemical.

(2) [Reserved]

(3) *Range-finding test.* A range-finding test should be conducted to establish test chemical concentrations for the definitive test.

(4) *Definitive test.* (i) A definitive test is used to determine time-growth and concentration-response curves, as well as the $EC_{50}$ and no effect level ($EC_0$) of the test chemical. The testing method will vary slightly depending upon whether the chemical is readily water-soluble, of limited solubility, or volatile.

(ii) Criteria for a valid definitive test. (A) Algae in the control flasks should exhibit log phase growth within 48 hours of test initiation and should produce a standing crop of at least $10^5$ cells/ml in 96 hours.

(B) At 96 hours, one test concentration should show no significant decrease in growth rate and one concentration should show greater than a 50 percent decrease in growth rate relative to the control.

(C) The pH of each test solution should be measured before use and, if necessary, adjusted to 7.5 ± 0.2 using HCl or NaOH.

(D) The test chemical concentration should be determined before and after the test when practical.

(E) No more than 20 percent of the test chemical should be lost by volatilization. If more is lost (or is likely to be), the test should be conducted in closed flasks with a resulting lower standing crop.

(iii) *Test procedures dependent on solubility*—(A) *Readily water-soluble chemicals.* When readily water-soluble chemicals are tested, the following procedures are recommended:

(*1*) A stock solution of the readily water-soluble chemical should be prepared with micropore-filtered (0.45 μm pore size) medium. The concentration of the stock solution should be twice as high as the highest concentration to be used in the test. From this stock solution at least five dilutions should be made. The dilutions should be in a geometric series with a ratio of 10 or n/10 (where n=number of dilutions). A minimum of five concentrations should be used such that the highest concentration results in at least 50 percent growth inhibition and the lowest concentration shows no significant difference ($p = 0.05$) from the control.

(*2*) The algal suspension for testing readily-soluble chemicals should have a density of approximately $2 \times 10^4$ cells/ml of medium. The cell density should be quantified prior to beginning the test.

(*3*) The test should be carried out in triplicate (i.e., 3 replicate flasks per concentration). The test begins by transferring 50 ml of algal suspension to 250 ml Erlenmeyer flasks. Then, in sequence of increasing concentrations, a 50 ml volume of each prepared dilution is added to the appropriate flask. The control flasks receive 50 ml of medium. The flasks are then gently shaken and placed in the test chambers. The algal concentration in samples from each flask is determined at intervals of at least 24, 48, 72, and 96 hours after the start of the test and the number of cells or biomass (dry weight) per ml is calculated for each sample.

(*4*) A fluorimeter or spectrometer can be used to calculate cell number or biomass, but will not provide precise measurements at the start of the test and at 24 hours. If a Coulter Counter, or spectrophotometer, is used to enumerate algae at the beginning of the test, 100 ml of medium should be used to determine the background.

(B) *Limited water-soluble chemicals.* When chemicals with limited water-solubility are tested, the following modifications of the above procedure are recommended:

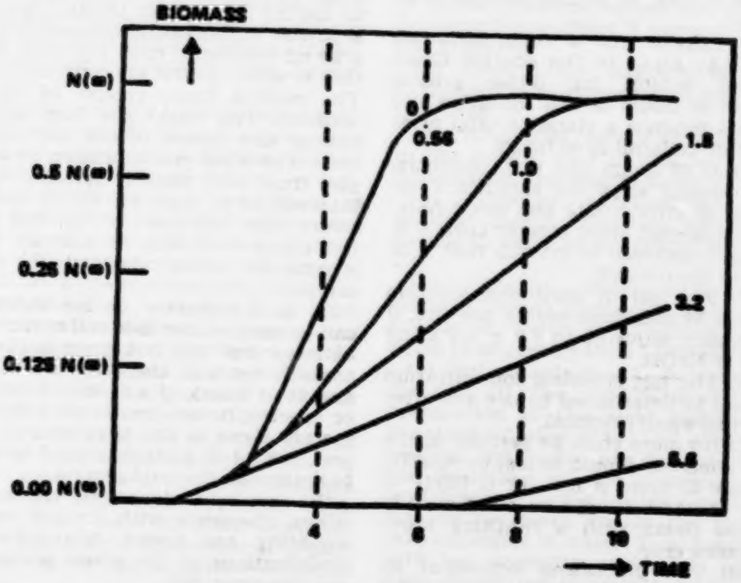
(*1*) A stock solution for chemicals with limited water-solubility should be prepared with a suitable organic solvent. This stock solution should be $10^4$ times as concentrated as the highest concentration to be tested and the amount of solvent necessary to dissolve the chemical should not exceed 0.1 ml/l at the highest chemical concentration used.

(*2*) An algal suspension for testing chemicals with limited water-solubility should have a density of approximately $10^4$ cells/ml. One hundred (100) ml of this algal suspension ($10^4$ cells/ml) are placed in each flask and 10 μl of the various dilutions of test chemical and solvent are added. Ten (10) μl of solvent are added to the solvent control flasks. Otherwise the test should be conducted as described for readily-soluble chemicals.

(iv) *Test procedures dependent on volatility.* When volatile chemicals are tested, the following modification of the procedures in paragraphs (c)(4)(iii) (A) and (B) of this section is recommended. The modification prescribes the use of 250 ml conical flasks with ground glass stoppers instead of 250 ml Erlenmeyer flasks prescribed in paragraph (c)(4)(iii)(A)(*3*) of this section. These flasks are filled as described above with the algal suspension and test chemical solution. For each measurement interval the complete contents of the flasks should be used. Therefore, it is necessary to use a sufficient number of flasks to allow for this destructive sampling with time.

(5) *Test results.* (i) The results of the measurements should be tabulated and plotted on semi-logarithmic paper to yield growth curves as illustrated in the following Figure 1:

Figure 1—Theoretical Example of a Result of a Toxicity Test With Algae



The growth curve is plotted on a simple logarithmic scale. The figures give the concentrations of the test compound in weight per volume. The broken lines show the measurements. $N(\infty)$ is the maximum cell density that can be achieved.

(ii) The reported chemical effects on algae should include both the $EC_{50}$ value and the no-effect level ($EC_0$). A number of methods have been described for determining the $EC_{50}$ and $EC_0$. Two examples are provided.

(A) *$EC_50$ and $EC_0$ determined from the specific maximum growth rate* ($\mu$). The mean value of the three replicate measurements and the 95 percent confidence limits should be calculated for the 24, 48, 72, and 96 hour sampling times, and plotted as in Figure 1. Two values are selected from the log-linear portion of the curve and the specific growth rate ($\mu$) is calculated according to the following formula:

$$\mu = \frac{\log_{10}(N_{1/N2})}{0.434(t_2 - t_1)}$$

where:

$N_1$ = the lower cell number chosen in the log phase at $t_1$

$N_2$ = the higher cell number chosen in the log phase at $t_2$

t = time in hours

u = maximum specific growth rate

0.434 = coefficient to convert $\log_{10}$ data to $\log_e$

The percent reduction of the growth rate compared to the control can be calculated:

$$\text{"\% inhibition"} = \frac{1 - u(tox)}{u(b)} \times 100$$

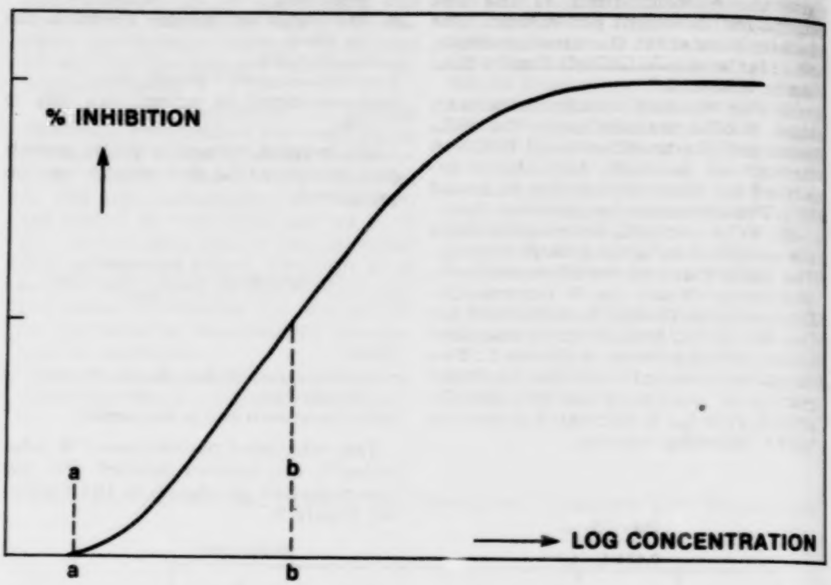
where:

u(tox) = the growth rate in the presence of the chemica

u(b) = the growth rate in the control

The calculated percentages ("% inhibition") are plotted against the log concentration as shown in the following Figure 2:

FIGURE 2—THEORETICAL EXAMPLE OF THE RELATION BETWEEN THE LOGARITHM OF THE CONCENTRATION OF THE CHEMICAL AND THE PERCENTAGE INHIBITION



The highest concentration showing no difference from the control (*1*) and the $EC_{50}$ (*2*) can then be read from this graph.

(B) *$EC_{50}$ determined from the mean relative growth rate.* (*1*) The mean relative growth rate (RGR) during log phase growth for each culture can be calculated as follows:

$$RGR = \frac{\mathrm{Log}_{10}n_2 - \mathrm{Log}_{10}n_1}{t_2 - t_1}$$

where:

$n_1$ = number of cells/ml at $t_1$
$n_2$ = number of cells/ml at $t_2$
t = time (hours).

(*2*) The RGR of the three control replicates can be calculated and its 95 percent confidence limits determined. Treatment RGR values that are greater than the control upper 95 percent confidence limit indicate algal stimulation and should be ignored. Those above the control RGR but within the 95 percent confidence interval should be assumed to equal the control mean. Treatment RGR values that are less than the lower 95 percent confidence limit of the control should be used in the following calculations: The low treatment RGR values should be expressed as a percentage of the control RGR. The percentage values including the highest concentration of test compound at which growth is 100 percent that of control (zero inhibition and the $EC_0$) and the lowest concentration with a value of 0 percent (100 percent inhibition) should be included in an appropriate statistical analysis to determine the $EC_{50}$ value and its 95 percent confidence limits.

(*3*) Interpretation of results. Algal populations rapidly regenerate themselves upon removal of stress; consequently, concentrations that produce effects need careful interpretation.

(6) [Reserved]

(d) *Test conditions*—(1) *Test species*—(i) *Selection.* (A) It is recommended that the algae used be a fast-growing species that is convenient for culturing and testing. The following freshwater species are considered suitable:

(*1*) *Selenastrum capricornutum.*

(*2*) *Scenedesmus quadricauda.*

(*3*) *Chlorella vulgaris.*

(B) Axenic cultures are recommended and are highly desirable when testing biodegradable compounds. However, pure monocultures of algae are required.

(ii) *Stock culture.* (A) The stock cultures are algal cultures that are regularly transferred to fresh medium to act as initial test material. Cultures that are not used regularly should be streaked out on sloped agar tubes. The tube cultures should be transferred to fresh medium at least once every 2 months.

(B) The stock cultures should be grown in Erlenmeyer flasks containing the appropriate medium (volume about 100 ml). When the algae are incubated at 20 °C with continuous illumination, a weekly transfer is recommended. An amount of "old" culture is transferred with sterile pipettes into a flask of fresh medium, for an approximate 100-fold dilution. The growth rate of a species can be determined from the growth curve. If this is known, it is possible to estimate at what density the culture should be transferred to new medium. This should be done before the culture reaches the senescent phase.

(iii) *Selection of test algae.* (A) Algae used in a test should be in an exponential growth phase, with a cell density of at least $10^4$ cells/ml. If it is not possible to use the stock culture directly for testing (due to lack of or excessive cell growth) it may be necessary to pre-culture the algae prior to use.

(B) To preculture the algae, 2 liters of sterile nutrient solution are added to a 3 liter Erlenmeyer flask. Sufficient algal suspension from the stock culture is added to give an algal concentration of approximately $10^4$ cells/ml (± 25 percent). The flasks should be continuously illuminated with fluorescent light, and the temperature maintained at 20 °C. Cultures should be shaken by hand at least once every day; this is particularly important for non-motile species. The cell concentration in the culture should be determined daily so that the desired concentration may be obtained for testing.

(C) Other methods of culturing may also be used. Some algae can be grown rapidly in shake cultures. Bubbling with air containing additional carbon dioxide may also accelerate growth. Furthermore, under the culturing conditions described above, the pre-culture, for the algal species recommended in this test, should be optimal for inoculation of test flasks after 4 to 5 days. If the algal cultures contain deformed or otherwise abnormal cells (e.g., clumped, chlorotic), they should not be used for the test.

(2) *Facilities*—(i) *Apparatus.* (A) In order to avoid contamination with bacteria and other algae, all stock culture maintenance operations should be carried out under sterile conditions. Possible contamination should be checked by suitable methods.

(B) Testing and culturing of algae should be done in an environmentally controlled cabinet or chamber capable of maintaining a temperature of 20 °C ± 2 °C and continuous illumination of approximately 300 to 400 $uE/m^2 sec$.

(C) In addition to normal laboratory apparatus and equipment for algal testing, a counting apparatus (e.g., Coulter Counter, counting chamber, fluorometer, spectrophotometer, colorimeter) to determine cell numbers is also necessary.

(D) The following apparatus and equipment are necessary for algal culturing:

(*1*) Incubators or climate rooms capable of maintaining temperature and light at the recommended levels.

(*2*) Filtering apparatus, accompaning membrane filters, (0.45 μm) and 5 liter flasks.

(*3*) Inoculation needle.

(*4*) Sterile graduated pipettes.

(*5*) pH-meter.

(*6*) Culture tubes (150 × 18 mm) with sponge and/or metal caps.

(*7*) Erlenmeyer flasks, 200 ml, with ground glass stoppers, and a 3 liter flask.

(ii) *Dilution water.* Redistilled water should be used for preparation of stock, chemical, and nutrient media solutions.

(3) *Test parameters*—(i) *Carriers.* If solvents, are used, they should not themselves be toxic at the concentrations used and should not affect algal growth. In no case should the concentration of an organic solvent exceed 0.1 ml/1 in the highest concentration.

(ii) *Lighting.* Algae should be kept under continuous, uniform illumination of approximately 300 to 400 uE/m$^2$ sec. The light source should be fluorescent lights.

(iii) *Loading.* For readily water-soluble and volatile chemicals, an algal suspension containing approximately $2\times10^4$ cells/ml should be used. For chemicals with limited water solubility the algal density should be approximately $10^4$ cells/ml.

(iv) *Temperature.* The temperature for culturing and testing algae should be
20 °C ± 2 °C.

(v) *Nutrient media.* The recommended media for adequate growth of algae is shown in the following Table 1:

TABLE I—MEDIUM FOR FRESHWATER ALGAE

| | Amount |
|---|---|
| *Nutrient salts:* | |
| $NaNO_3$ | 0.5 g. |
| $NH_4NO_3$ | 0.33 g. |
| $K_2HPO_4.3H_2O$ | 0.052 g. |
| $CaCl_2.2H_2O$ | 0.035 g. |
| $Na_2CO_3.10H_2O$ | 0.054 g. |
| $Fe_2$-citrate | 0.006 g. |
| Citric acid monohydrate | 0.006 g. |
| Trace element solution (see below). | 1.0 ml. |
| Redistilled water made up to | 1.0liter. |
| pH | 7.7 ±0.3. |
| *Trace Element Solution:* | |
| $H_3BO_3$ | 2.90 g. |
| $MnCl_2.4H_2O$ | 1.81 g. |
| $ZnCl_2$ | 0.11 g. |
| $CuSO_4.5H_2O$ | 0.08 g. |
| $(NH_4)_6Mo_7.4H_2O$ | 0.018 g. |
| Redistilled water made up to | 1.0 liter. |

All nutrient solutions can be prepared as concentrated stock solutions using the salts shown in Table 1 under this paragraph and stored in the dark and cold. These solutions should be sterilized by filtration or by autoclaving. The medium is prepared by adding the correct amount of stock solutions, or the nutrients salts directly, to sterile distilled water, to give the final concentrations listed. For solid medium, 1.5 to 2 percent agar can be added. Other media may be necessary if species other than those above are used.

(e) *Reporting.* In addition to a description of the type of test and method, the report submitted to EPA should include the following information.

(1) For the chemical tested: Manufacturer, empirical formula, batch number and its degree of purity, chemical characterization (e.g., trade name), and physical properties.

(2) For the test organisms: Origin of innoculum, laboratory culture and strain number, and method of cultivation (including whether cultures aerated and/or shaken).

(3) For the test conditions: Date of beginning and end of the test and its duration, temperature, light intensity and light quality in the growth chamber, type of test flask (and if closed or open), initial pH of test solution, what carrier is used and how much, concentrations of test chemical, and the counting method.

(4) For the results: A tabulation of cell number or biomass per ml for each flask at each sampling period, the plotted time-growth curves for each concentration and the concentra-

tion-effect curve, the $EC_{50}$ value and the highest concentration showing no statistical growth inhibition ($EC_0$) and the statistical methods used to calculate them, and other observed effects, e.g. algicidal vs. algistatic effects, clumping or chlorosis of cells.

(5) For the laboratory performing the test: The name of the person responsible for carrying out the test (study director) as well as the name of the person carrying out the test, the name and address of the testing laboratory, and the date and signature of the person responsible for the test.

[50 FR 39321, Sept. 27, 1985, as amended at 53 FR 12526, Apr. 15, 1988]

## § 797.1075 Freshwater and marine algae acute toxicity test.

(a) *Purpose.* (1) A unicellular alga is used as a model system initially to estimate the concentration of a chemical which could affect survival and growth of freshwater or marine algae. Because generation times for unicellular algal species are measured in hours, this relatively short test can assess effects over several generations. Results allow the assessment of effects on initial organisms from short term exposures and give an indication of the effect on algal populations.

(2) Many different protocols for algal tests are available. This growth test is easy to perform and gives reproducible results with the recommended species. This test guideline can be adapted for other algal species. If such an adaptation is used, a description of the method should be provided with the test report.

(b) *Definitions.* The following definitions apply to this guideline:

(1) "EC-X" means the experimentally derived chemical concentration that is calculated to effect X percent of the test criterion.

(2) "Growth rate" means the rate at which the algal population grows, estimated by the increase in cell numbers or biomass over a specified period of time.

(3) "Inhibition" means any decrease in the growth rate of the population of test algae compared to the controls.

(4) "Limited water-soluble substances" means chemicals which are soluble in water at less than 1,000 mg/l.

(5) "Readily water-soluble substances" means chemicals which are soluble in water at a concentration equal to or greater than 1,000 mg/l.

(c) *Test procedures*—(1) *Summary of the test.* (i) The procedures for the preparation of the algal suspension, the stock solution of the test chemical, and the test media, are dependent on the solubility of the chemical. Modifications in the testing procedure may be necessary due to the chemical's solubility in water. For chemicals with low solubility under test conditions, it may not be possible to quantitatively determine the $EC_{50}$.

(ii) For purposes of the test, algae are grown in Erlenmeyer flasks in an environmentally controlled growth chamber. The test is started when an algal suspension in nutrient solution ($10^4$ or $2 \times 10^4$ cells/ml) and the appropriate test chemical dilutions are placed in the flasks. Algal growth is measured at 24-hour intervals for at least 96 hours. A Coulter Counter, cell counting chamber, or other appropriate instruments may be used to determine population density. The data are used to define the concentration-response curve and the time-growth curve, and to calculate the $EC_{50}$ and $EC_0$ (no-effect level) for the chemical.

(2) [Reserved]

(3) *Range-finding test.* A range-finding test should be conducted to establish test chemical concentrations for the definitive test.

(4) *Definitive test.* (i) The definitive test is used to determine time-growth and concentration-response curves, as well as the $EC_{50}$ and $EC_0$ of the test chemical. The testing method will vary slightly depending upon whether the chemical is readily water-soluble, of limited solubility, and/or volatile.

(ii) Criteria for a valid definitive test. (A) Algae in the control flasks should exhibit log phase growth within 48 hours of test initiation and should produce a population density of at least $10^5$ cells/ml in 96 hours.

(B) At 96 hours, one test concentration should show no significant decrease in growth rate and one concentration should show greater than a 50 percent decrease in growth rate relative to the control.

(C) The pH of each test solution should be measured before use and, if necessary, adjusted to 7.5 ± 0.2 for freshwater and 8.0 ± 0.1 for marine species using HCl or NaOH.

(D) The concentration of the test chemical in the medium should be determined before and after the test.

(E) No more than 20 percent of the test chemical should be lost by volatilization. If more is lost (or is anticipated to be), the test should be conducted in closed flasks. Carbon dioxide limitations resulting from the closed flasks typically result in population densities lower than the standard in paragraph (c)(4)(ii)(A) of this section.

(iii) Test procedures dependent on solubility—(A) *Readily water-soluble chemicals.* When readily water-soluble chemicals are tested, the following procedures are recommended:

(*1*) Just prior to testing, a stock solution of the readily water-soluble chemical should be prepared with micropore-filtered (0.45 um pore size) medium. The concentration of the stock solution should be twice as high as the highest concentration to be used in the test. From this stock solution at least five dilutions should be made. The dilutions should be in a geometric series with a ratio of 10 or n/10 (where n=number of dilutions). The highest concentration should result in at least 50 percent growth inhibition while the lowest concentration should result in no significant difference ($\alpha=0.05$) from the growth in the controls.

(*2*) The algal suspension for testing readily-soluble chemicals should have a density of approximately $2\times10^4$ cells/ml of nutrient solution. The cell density should be quantified prior to beginning the test.

(*3*) The test should be carried out in triplicate (i.e., three replicate flasks per concentration). The test begins by transferring 50 ml of algal suspension to 250 ml Erlenmeyer flasks. Then, in sequence of increasing concentrations, a 50 ml volume of each prepared dilution is added to the appropriate flask. The control flasks receive 50 ml of nutrient solution. The flasks are then gently shaken and placed in the growth chambers. The flasks should be shaken by hand at least once every day; this is particularly important for nonmotile species. The algal concentration in samples from each flask is determined at intervals of at least 24, 48, 72, and 96 hours after the start of the test and the number of cells or biomass (dry weight per ml) is calculated for each sample.

(*4*) If a fluorometer or spectrometer is used to calculate cell number or biomass, the measurements will be inaccurate during the first 24 hours of the test period. Therefore, a calibration curve of meter readings of cell numbers versus test concentrations should be plotted. Calculations should be limited to the straight portion of the calibration curve.

(B) *Limited water-soluble chemicals.* When chemicals with limited water-solubility are tested, the following modifications of the above procedure are recommended:

(*1*) Just prior to testing, stock solutions of test chemicals should be prepared with a suitable organic solvent. Each stock solution should be $10^4$ times more concentrated than the highest concentration to be tested. The amount of solvent necessary to dissolve the chemical should not exceed 0.1 ml/l of stock solution.

(*2*) The algal suspension should have a density of approximately $10^4$ cells/ml. Otherwise the test should be conducted as described for readily-soluble chemicals.

(*3*) Each test flask should contain 100 ml of algal suspension and a 10 $\mu$l aliquote of the diluted chemical stock. A series of dilutions is made of the chemical stock so that when 10 $\mu$l of each dilution is combined with 100 ml of algal suspension the desired test concentrations are obtained. Two controls should be included, one possessing the solvent (10 $\mu$l of 0.1 ml/l) and no test chemical, and the second without either.

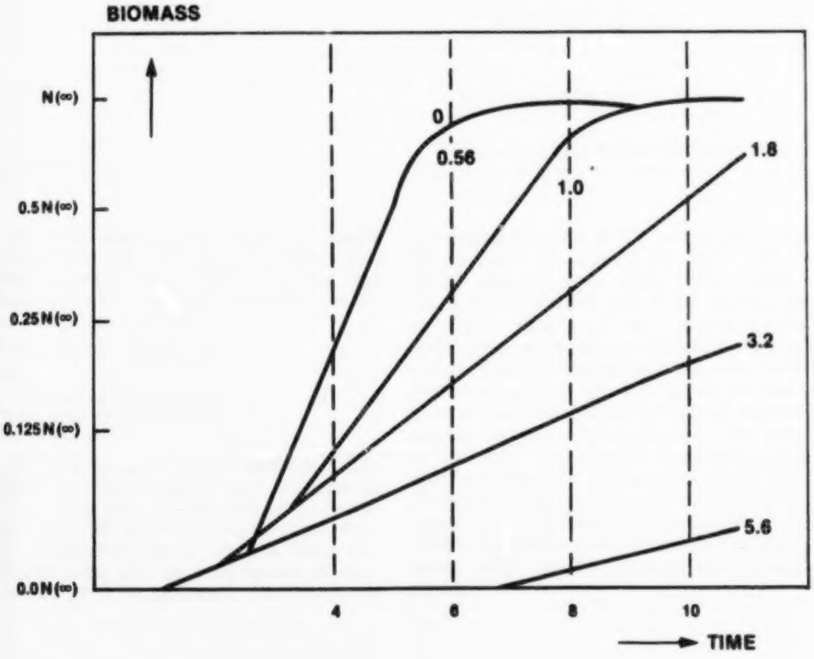
(iv) Test procedures dependent on volatility. When volatile chemicals are tested, the following modifications of the procedures in paragraphs (c)(4)(iii) (A) and (B) of this section are recommended. The test is performed as described above (depending on the chemical's solubility in water) except that 250 ml conical flasks with ground glass stoppers should be used. These flasks are filled, as described above, with the algal suspension and test chemical solution. For each measurement interval the complete contents of the flasks should be used. Therefore, it is necessary to use a sufficient number of flasks to allow for this destructive sampling with time.

(5) *Test results.* (i) The results of the measurements should be tabulated. Growth curves resulting from the experiment should be drawn on semi-logarithmic paper.

(ii) The reported chemical effects on algae should include both the $EC_{50}$ and $EC_0$. A number of methods have been described for determining the $EC_{50}$ and $EC_0$. Two examples are provided.

(A) *$EC_{50}$ and $EC_0$ determined from the specific maximum growth rate* (*u*). The mean value of the three replicate measurements and the 95 percent confidence limits should be calculated for the 24, 48, 72, and 96 hour sampling times, and plotted as in the following Figure 1:

FIGURE 1—THEORETICAL EXAMPLE OF A RESULT OF A TOXICITY TEST WITH ALGAE



The growth curve is plotted on a simple logarithmic scale. The figures give the concentrations of the test compound in weight per volume. The broken lines show the measurements. $N(\infty)$ is the maximum cell density that can be achieved.

Two values are selected from the log-linear portion of the curve and the specific growth rate (u) is calculated according to the following formula:

$$u=\frac{\ln (N_n/N_1)}{(t_n-t_1)}$$

where:

$N_1$=the lower cell number chosen in the log phase at $t_1$

$N_n$=the higher cell number chosen in the log phase at $t_n$

t=time in hours

u=maximum specific growth rate

The percent reduction of the growth rate compared to the control can be calculated:

"% inhibition"$=1-(u(tox)/(u(b)))\times 100$

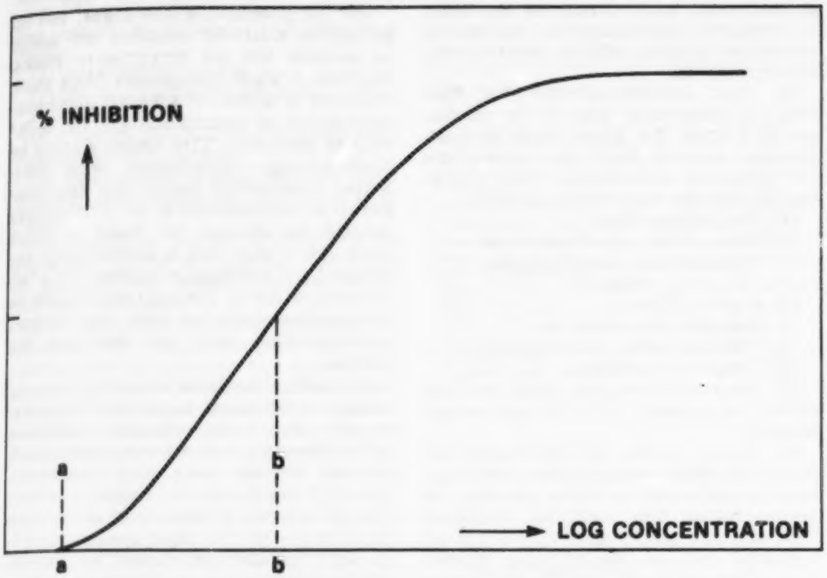
where:

u(tox)=the growth rate in the presence of the chemical

u(b)=the growth rate in the control

The calculated percentages ("% inhibition") are plotted against the log concentration as shown in the following Figure 2. The highest concentration showing no difference from the control (*1*) and the $EC_{50}$ (*2*) can then be read from this graph.

FIGURE 2—THEORETICAL EXAMPLE OF THE RELATION BETWEEN THE LOGARITHM OF THE CONCENTRATION OF THE CHEMICAL AND THE PERCENTAGE INHIBITION



(B) *$EC_{50}$ determined from the mean relative growth rate.* (*1*) The mean relative growth rate (RGR) during log phase growth for each culture can be calculated as follows:

$RGR = (\log_{10} n_2 - \log_{10} n_1)/(t_2 - t_1)$

where:

$n_1$ = number of cells/ml at $t_1$
$n_2$ = number of cells/ml at $t_2$
t = time (hours)

(*2*) The mean relative growth rates (RGR) of the controls and treatment along with the 95 percent confidence intervals (95 percent CI) are calculated. Treatment RGR values that are greater than the control upper 95 percent CI will be ignored. Those above the mean control RGR but within the 95 percent CI should be assumed to equal the control mean. The treatment RGR values are then expressed as a percentage of the control mean RGR. The percentage values including the highest concentration of test compound at which growth is 100 percent that of control (if values for all higher concentrations are less than 100 percent) and the lowest concentration with a value of 0 percent (if values for higher concentrations are less than or equal to 0 percent) are included in a probit analysis to determine the $EC_{50}$ value with its 95 percent confidence limits.

(*3*) *Interpretation of results.* Algal populations subjected to chemical stress may quickly establish normal growth rates when the stress condition is removed; consequently, concentrations that produce effects need careful interpretation.

(*d*) *Test conditions*—(1) *Test Species*—(i) *Selection.* (A) It is recommended that the algae used be fast-growing species that are convenient for culturing and testing. The following species are considered suitable:

(*1*) *Freshwater Algae*—

(*i*) *Selenastrum capricornutum.*

(*ii*) *Scenedesmus quadricauda.*

(*iii*) *Chlorella vulgaris.*

(*2*) *Marine Algae*—

(*i*) *Skeletonema costatum.*

(*ii*) *Thallassiosira pseudonana.*

(*iii*) *Isochrysis galbana.*

(B) Monocultures of algae are required, and axenic cultures are recommended.

(ii) *Stock culture.* (A) The stock cultures are algal cultures that are regularly transferred to fresh medium to act as initial test material. Cultures that are not used regularly should be streaked out on sloped agar slants. The tube cultures should be transferred to fresh medium at least once every 2 months. Stock cultures taken from agar slants should be subcultured in liquid medium once or twice before use.

(B) The stock cultures should be grown in Erlenmeyer flasks containing the appropriate medium (volume about 100 ml). When the algae are grown at 20 °C under continuous cool-white fluorescent light, a weekly transfer is recommended. An aliquot removed from a mature culture transferred with a sterile pipette into a flask of fresh medium, for an approximate 100-fold dilution. The growth rate of a species can be determined from the growth curve. If this growth rate is known, it is possible to estimate at what density the culture should be transferred to new medium. This should be done before the culture is out of log growth.

(iii) *Selection of test algae.* (A) Algae used in a test should be in an exponential growth phase, with a cell density of at least $10^4$ cells/ml. If it is not possible to use the stock culture directly for testing (due to lack of or excessive cell growth) it may be necessary to preculture the algae prior to use.

(B) To preculture the algae, 200 ml of sterile nutrient solution are added to several 500 ml Erlenmeyer flasks. Sufficient algal suspension from stock cultures is added to give an algal concentration of approximately $10^4$ cells/ml±25 percent). The flasks should be continuously illuminated with cool-white fluorescent light, and the temperature maintained at 20 °C. Cultures should be shaken by hand at least once every day; this is particularly important for nonmotile species. The cell concentration in the culture should be determined daily so that the desired concentration may be obtained for testing.

(C) Other methods of culturing may also be used. Some algae can be grown rapidly in shake cultures. Bubbling with filtered air containing additional carbon dioxide may also accelerate growth. Furthermore, under the culturing conditions described above, the pre-culture for the algal species recommended in this test, should be optimal for inoculation of test flasks after 4 to 5 days. If the algal cultures contain deformed or otherwise abnormal cells (e.g., clumped, chlorotic), they should not be used for the test.

(2) *Facilities*—(i) *Apparatus.* (A) In order to avoid contamination with bacteria and other algae, all stock culture maintenance operations should be carried out under aseptic conditions. Cultures should be periodically checked for contamination.

(B) Testing and culturing of algae should be done in an environmentally controlled cabinet or chamber capable of maintaining a temperature of 20 °C±2 °C and continuous illumination of approximately 150 to 200 $\mu$E/m$^2$ sec.

(C) In addition to normal laboratory apparatus and equipment for algal testing, a counting apparatus (e.g., Coulter Counter, cell counting chamber, fluorometer, spectrophotometer, or colorimeter) to determine cell numbers is also necessary.

(D) The following apparatus and equipment are necessary for algal culturing:

(*1*) Incubators or climate rooms capable of maintaining temperature and light at the recommended intensity.

(*2*) Filtering apparatus, accompanying membrane filters, (0.45 um) and flasks.

(*3*) Inoculation needle.

(*4*) Sterile graduated pipettes.

(*5*) pH-meter.

(*6*) Culture tubes (150×18 mm) with stainless steel closures.

(*7*) Erlenmeyer flasks, 250 ml, with ground glass stoppers, and a 3 liter flask.

(ii) *Dilution water.* Redistilled or deionized water should be used for preparation of stock, chemical, and nutrient solutions.

(3) *Test parameters*—(i) *Carriers.* If solvents, are used, they should not themselves be toxic at the concentrations used and should not affect algal growth. In no case should the concentration of an organic solvent exceed 0.1 ml/l in the highest concentration.

(ii) *Lighting.* Algae should be kept under continuous, uniform illumination of approximately 150 to 200 $\mu$E/m² sec. The light source should be cool-white fluorescent lights.

(iii) *Inoculation.* For readily water-soluble and volatile chemicals, an algal suspension containing approximately $2 \times 10^4$ cells/ml should be used. For chemicals with limited water solubility the algal density should be approximately $10^4$ cells/ml. After dilution, both initial algal densities should be approximately $10^4$ cells/ml.

(iv) *Temperature.* The temperature for culturing and testing algae should be 20 °C ± 2 °C.

(v) *Nutrient media.* The recommended media for adequate growth of algae are shown in the following Table 1 and Table 2. All nutrient solutions can be prepared as concentrated stock solutions using the salts shown in the following Tables 1 and 2 and stored in the dark and cold. These solutions should be sterilized by filtration or by autoclaving. The medium is prepared by adding the correct amount of stock solutions, or the nutrient salts directly, to sterile distilled or deionized water, to give the final concentrations listed. For solid medium, 1.5 to 2 percent agar (wt/vol) should be added to the medium prior to autoclaving. Other media may be necessary if species other than those above are used.

TABLE 1—MEDIUM FOR FRESHWATER ALGAE

| | Amount |
|---|---|
| Solution 1 (Nutrient salts): | |
| $NaNO_3$ | 0.5 g |
| $NH_4NO_3$ | 0.33 g |
| $K_2HPO_4.3H_2O$ | 0.052 g |
| $CaCl_2.2H_2O$ | 0.035 g |
| $Na_2CO_3.10H_2O$ | 0.054 g |
| $Fe_3$-citrate | 0.006 g |
| Citric acid monohydrate | 0.006 g |
| Trace element solution (sol. 2) (see below) | 1.0 ml |
| Redistilled water made up to | 1.0 liter |
| pH | 7.7±0.2 |
| Solution 2 (Trace Metals): | |
| $H_3BO_3$ | 2.90 g |
| $MnCl_2.4H_2O$ | 1.81 g |
| $ZnCl_2$ | 0.11 g |
| $CuSO_4.5H_2O$ | 0.08 g |
| $(NH_4)_6MO_7.4H_2O$ | 0.018 g |
| Redistilled water made up to | 1.0 liter |

TABLE 2—MEDIUM FOR MARINE ALGAE

| | mmol/1 | g/kg solution |
|---|---|---|
| Solution 1 (Nutrient salts) | | |
| NaCl | 362.661 | 20.758 |
| $Na_2SO_4$ | 24.993 | 3.477 |
| KCl | 8.038 | 0.587 |
| KBr | 0.7249 | 0.0845 |
| $H_3BO_3$ | 0.3715 | 0.0225 |
| NaF | 0.0657 | 0.0027 |
| $MgCl_2.6H_2O$ | 47.176 | 9.395 |
| $CaCl_2.2H_2O$ | 9.139 | 1.316 |
| $SrCl_2.6H_2O$ | 0.0082 | 0.0214 |
| Solution 2 | | |
| $NaHCO_3$ [1] | 2.066 | 0.170 |
| Solution 3 (Trace Metals) [3] | g/l | umol/1 [2] |
| $NaNO_3$ | 4.667 | 549.09 |
| $Na_2SiO_3{\cdot}9H_2O$ [4] | 3.000 | 105.60 |
| $Na_2$ glycerol $PO_4$ | 0.667 | 21.79 |
| $Na_2EDTA$ [3] | 0.553 | 14.86 |
| $H_3BO_3$ | 0.380 | 61.46 |
| $Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$ | 0.234 | 5.97 |
| $FeCl_3{\cdot}6H_2O$ | 0.016 | 0.592 |
| $MnSO_4{\cdot}4H_2O$ | 0.054 | 2.42 |
| $ZnSO_4{\cdot}7H_2O$ | 0.0073 | 0.254 |
| $CoSO_4{\cdot}7H_2O$ | 0.0016 | 0.0569 |
| Solution 4 (Vitamins [3]) | | |
| Thiamine HCl | 0.1 | 0.297 |
| Vitamin $B_{12}$ | 0.002 | 0.00147 |
| Biotin | 0.001 | 0.00409 |

[1] Dry, autoclave the $NaHCO_3$ then add to autoclaved solution 1.

[2] Final concentration in solution.

[3] Should be autoclaved (50 minutes).

[4] Should be neutralized before adding to other solutions. To neutralize, 100 ml of $Na_2SiO_3{\cdot}9H_2O$(3.0 g/l) is added to 20 ml 1 *N* HCl and then added to solution.

[3] $Na_2$ EDTA, $FeCl_3 \cdot 6H_2O$, $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ has to be in solution before remaining salts (Mn, Zn, Co) are added.

[4] Filter, sterilize, store frozen.

(e) *Reporting.* In addition to a description of the type of test and method, the report submitted to EPA should include the following information.

(1) For the chemical tested: Manufacturer, empirical formula, batch number and its degree of purity, chemical characterization, and physical properties.

(2) For the test organisms: Origin of innoculum, laboratory culture and strain number, and method of cultivation (including whether and how cultures were aerated and/or shaken).

(3) For the test conditions: Date of beginning and end of the test and its duration, temperature, light intensity and light quality in the growth chamber, type of test flask (and if closed), initial pH of test solution, what carrier was used and how much, concentrations of test chemical, and the counting method.

(4) For the results: A tabulation of cell number or biomass (dry weight per ml) for each flask at each sampling period; the plotted time-growth curves for each concentration and the concentration-effect curve; the $EC_{50}$ and $EC_5$ values and the statistical methods used to calculate them; and other observed effects, e.g., stimulation of growth, algicidal vs. algistatic effects, clumping or chlorosis of cells.

(5) For the laboratory performing the test: the name of the person responsible for carrying out the test (e.g., study director) as well as the name of the person carrying out the test, the name and address of the testing laboratory, and the date and signature of the person responsible for the test.

### § 797.1160 Lemna acute toxicity test.

(a) *Purpose.* This guideline prescribes test procedures and conditions using the freshwater aquatic plant *Lemna gibba* G3 to develop data on the phytotoxicity of chemicals. The United States Environmental Protection Agency (USEPA) will use data from these tests in assessing the hazard of a chemical to the environment under the Toxic Substances Control Act (TSCA) (Pub. L. 94-469, 90 Stat. 2003, 15 U.S.C. 2601 *et seq.*).

(b) *Definitions.* The definitions in section 3 of TSCA and Part 792—Good Laboratory Practice Standards of this chapter are applicable to this guideline. The following definitions also apply:

(1) "Axenic" means a culture of *Lemna* fronds free from other organisms.

(2) "Colony" means an aggregate of mother and daughter fronds attached to each other.

(3) "EC-X" means the experimentally derived chemical concentration that is calculated to effect X percent of the test criterion.

(4) "Frond" means a single *Lemna* "leaf-like" structure.

(5) "Frond mortality" means dead fronds which may be identified by a total discoloration (yellow, white, black or clear) of the entire frond.

(6) "Static-replacement test" means a test method in which the test solution is periodically replaced at specific intervals during the test.

(c) *Test procedures*—(1) *Summary of the test.* (i) In preparation for the test, containers are filled with appropriate volumes of nutrient medium and/or the test solutions. The test is started by introducing *Lemna* fronds into each of the containers. Nutrient medium and test solutions should be replaced on days 3 and 6 or as needed to prevent nutrient limitation or depletion of the test chemical.

(ii) Colonies should be inspected for changes in frond number and appearance at the beginning of the exposure period (day 0), on days 3 and 6, and at the end of the exposure period (day 7). On day 7, the total number of living and/or dead fronds are counted. Any frond which is visible as a bud when viewed under a hand lens or dissecting microscope should be counted. Concentration response curves are plotted for total frond number, growth rate (as number of fronds per day) and mortality (percentage of dead fronds to total number of fronds). $EC_{10}$'s, $EC_{50}$'s, and $EC_{90}$'s are determined from the curves.

(2) [Reserved]

(3) *Range-finding test.* (i) A range-finding test should be conducted to es-

tablish (A) if definitive testing is necessary and (B) test solution concentrations for the definitive test.

(ii) The recommended procedure is to expose *Lemna* to a chemical concentration series (e.g., 0.1, 1.0, 10, 100, and 1000 mg/)l plus controls. Three, four-frond colonies should be selected and exposed to equal volumes of each chemical concentration for a period of seven days. The exposure period may be shortened if data suitable for the purpose of the range-finding test are obtained.

(iii) The lowest chemical concentration in a test series, exclusive of controls, should be the lowest concentration which can be analytically quantified. The highest concentration should be at least 1000 mg/l. Replicates are not needed and nominal concentrations of the chemical are acceptable for range-finding. If the calculated $EC_{50}$ is greater than 1000 mg/l or is less than the analytical detection limit, definitive testing is not necessary. However, replicates and measured concentrations of the appropriate dose are needed to substantiate this result.

(4) *Definitive test.* (i) The purpose of the definitive test is to determine the $EC_{10}$'s, $EC_{50}$'s, and $EC_{90}$'s for *Lemna* growth based on total frond number, growth rate and/or frond mortality with a minimum amount of testing beyond the range-finding test.

(ii) At least five concentrations of chemical, exclusive of controls, should be used in the definitive test. The concentration range should be selected to define the concentration response curve between the $EC_{10}$ and $EC_{90}$. For each concentration and control at least seven replicate containers should be used, each containing 150 ml of test solution and three randomly selected four-frond colonies. Fewer replicates, each containing a greater number of colonies, may be used; however, test containers and solution volumes will have to be adjusted accordingly. The range of chemical concentrations tested should result in the highest concentration affecting at least 90 percent of the fronds and the lowest concentration affecting no more than 10 percent of the fronds compared with the controls.

(iii) Every test should include controls consisting of the same nutrient medium, number of fronds, environmental conditions, and procedures as the test containers except that none of the chemical is added. If a solvent or carrier is used to dissolve or suspend the test chemical, additional controls containing the solvent or carrier should also be included in the test to determine any effect of the solvent or carrier on the plants.

(iv) The colonies should be transferred to freshly prepared test solutions on days 3 and 6. No more than 20 percent of the test substance should be lost by volatilization between replacements. The colonies may have to be transferred more frequently for highly volatile test substances in order to maintain 80 percent of the initial test substance concentration. Transfer should be done in a clean, draft-free area as quickly as possible to minimize contamination of the colonies.

(v) Observations of frond numbers and appearance should be made of the colonies on day 0, 3, 6, and 7. A dissecting microscope will facilitate observations.

(vi) Concentration response curves should be plotted. These curves can provide the basis for determining the $EC_{10}$'s, $EC_{50}$'s, and $EC_{90}$'s for total frond number, growth rate, and mortality.

(vii) Any change in frond development or appearance such as increase in number (a frond is counted regardless of size as long as it is visible adjacent to the parent frond), necrosis, chlorosis, etc., should be reported. Chlorophyll content of the colonies may be used as an indicator of chlorosis. Any additional observations such as sedimentation of test solution, sinking of fronds, or other abnormalities should also be recorded.

(viii) A randomized complete block design is recommended for the definitive test with blocks delineated within the test chamber. If, for any reason, blocking is not feasible, total randomization within chambers is suggested.

(5) [Reserved]

(6) *Analytical measurements*—(i) *Chemical.* Stock solutions should be prepared just prior to use and diluted with glass distilled or deionized water

to obtain the test solutions. Standard analytical methods, if available, should be used to establish concentrations of these solutions and should be validated before beginning the test. An analytical method is not acceptable if likely degradation products of the chemical, such as hydrolysis and oxidation products, give positive or negative interference. The pH of the test solutions should also be measured prior to and after use.

(ii) *Numerical.* The number of fronds is counted at the end of the definitive test. Means and standard deviations are calculated and plotted for each treatment and control. Appropriate statistical analyses are used to provide a goodness-of-fit determination for the concentration response curves.

(d) *Test conditions*—(1) *Test Species.* The test species to be used in these tests is *Lemna gibba* G3. Axenic cultures may be obtained from laboratory cultures or commercial sources. A stock culture grown from a single isolated frond should be used to inoculate all the flasks used in a given test.

(2) *Acclimation.* Axenic stock cultures should be grown in the aquaria for 2 weeks (with necessary transfers) prior to being used in a test. Four-frond plants used in a test should be randomly selected from the culturing tank. Inocula should be taken from cultures which are less than 2 weeks old.

(3) *Facilities*—(i) *Apparatus.* (A) A controlled environment growth chamber or an enclosed area capable of maintaining the specified test parameters (see paragraph (d)(4) of this section) is needed.

(B) Laboratory facilities for the mixing and diluting of nutrient medium and a source of distilled or deionized water are needed. An autoclave (for sterilizing glassware and media) and a sterile transfer hood (for maintenance of an axenic *Lemna* culture) are also necessary. Disposal facilities should be adequate to accommodate spent test solutions and plant materials as well as any bench covering, lab clothing, or other contaminated materials.

(ii) *Containers and support media.* Test containers may be glass beakers or Erlenmeyer flasks, large enough to hold 150 ml of test solution and the *Lemna* colonies without crowding for the duration of the test; 250 ml beakers are recommended. All containers should be of the same type and size. If fewer replicates are used, larger containers may be necessary to hold the additional colonies and test solution volume. The ratio of test solution to container volume should not exceed 3:5. For each test concentration and control the same number of replicates should be used.

(iii) *Cleaning and sterilization.* All glassware and equipment should be cleaned following good laboratory practice. The Nytex screen or inoculating loops used for transferring the *Lemna* should be disposed of after use or thoroughly cleaned and sterilized before reuse.

(iv) *Nutrient media.* Hoagland's nutrient medium is recommended for maintaining *Lemna* cultures and for use as the diluent in the preparation of various concentrations of test solutions. Deionized or distilled water should be used to make the nutrient medium. The medium should be freshly prepared prior to each transfer of *Lemna* cultures and for the preparation of new test solutions during the course of a test. The pH of the medium should be adjusted to between 4.8 to 5.2 by the addition of 0.1N NaOH for culture maintenance prior to addition of the test chemical. Nutrient media should not contain EDTA, other chelating agents, or organic metabolites such as sucrose.

(v) *Carriers.* Stock solutions of substances of low aqueous solubility may be prepared by use of organic solvents, emulsifiers or dispersants of low phytotoxicity to plants. When a solvent or carrier is used, a second set of controls should contain the same concentration of the solvent or carrier as that used in the highest concentration of the test substance. The concentration of the solvent or carrier should not exceed 0.1 ml/1.

(4) *Test parameters.* Environmental conditions should be maintained as specified below:

(i) Temperature at 25 ± 2 °C.

(ii) The pH of the nutrient medium between 4.8 and 5.2. Test solution pH may vary from the nutrient medium after the addition of the test chemical and/or carrier (if used). Any such changes should be recorded but not adjusted.

(iii) Continuous irradiation of 400±50 uEinsteins/$m^2$ sec measured at the test solution or nutrient medium surface.

(e) *Reporting.* Reporting requirements of Part 792—Good Laboratory Practice Standards of this chapter apply to this guideline. The following data should also be reported.

(1) Source of *Lemna* and taxonomic verification.

(2) Description of test chambers, type of lights, size of beakers or flasks used, number of concentrations and replicates per concentration, number of colonies per replicate, solution volumes, physical parameters of growth chambers (e.g., humidity, temperature, and light intensity).

(3) The pH and concentration of the test chemical in the test solutions prior to use and discarding on day 3, 6, and 7.

(4) Number of fronds per test concentration and control at the end of the test, the percent inhibition and/or stimulation of growth rate, and percent frond mortality for each test concentration compared to controls.

(5) If the range-finding test showed that the highest concentration of the chemical tested (not less than 1000 mg/l had no effect on *Lemna*, report the results and measured concentrations and a statement that the chemical is not phytotoxic at concentrations less than 1,000 mg/l.

(6) If the range-finding test showed greater than a 50 percent effect with a test concentration below the analytical detection limit, report the results and a statement that the chemical is phytotoxic below the analytical detection limit.

(7) Means and standard deviations for frond number, growth rate, and percent frond mortality in each test concentration. In addition, concentration response curve(s) with 95 percent confidence limits delineated, goodness-of-fit determination, and $EC_{10}$'s, $EC_{50}$'s, and $EC_{90}$'s, identified.

(8) Methods and data records from chemical and numerical analyses including validation methods and quality assurance procedures.

### § 797.1300 Daphnid acute toxicity test.

(a) *Purpose.* This guideline is intended for use in developing data on the acute toxicity of chemical substances and mixtures ("chemicals") subject to environmental effects test regulations under the Toxic Substances Control Act (TSCA) (Pub. L. 94-469, 90 Stat. 2003, 15 U.S.C. 2601 *et seq.*). This guideline prescribes an acute toxicity test in which daphnids (*Daphnia magna* or *D. pulex*) are exposed to a chemical in static and flow-through systems. The United States Environmental Protection Agency will use data from this test in assessing the hazard a chemical may present in the aquatic environment.

(b) *Definitions.* The definitions in section 3 of the Toxic Substances Control Act (TSCA) and Part 792—*Good Laboratory Practice Standards* of this chapter apply to this test guideline. In addition, the following definitions apply to this guideline:

(1) "Brood stock" means the animals which are cultured to produce test organisms through reproduction.

(2) "$EC_{50}$" means that experimentally derived concentration of test substance in dilution water that is calculated to affect 50 percent of a test population during continuous exposure over a specified period of time. In this guideline, the effect measured is immobilization.

(3) "Ephippium" means a resting egg which develops under the carapace in response to stress conditions in daphnids.

(4) "Flow-through" means a continuous or an intermittent passage of test solution or dilution water through a test chamber or culture tank with no recycling.

(5) "Immobilization" means the lack of movement by the test organisms except for minor activity of the appendages.

(6) "Loading" means the ratio of daphnid biomass (grams, wet weight) to the volume (liters) of test solution in a test chamber at a point in time, or passing through the test chamber during a specific interval.

(7) "Static system" means a test system in which the test solution and test organisms are placed in the test chamber and kept there for the duration of the test without renewal of the test solution.

(c) *Test procedures*—(1) *Summary of the test.* (i) Test chambers are filled with appropriate volumes of dilution water. In the flow-through test, the flow of dilution water through each chamber is adjusted to the rate desired. The test chemical is introduced into each treatment chamber. The addition of test chemical in the flow-through system is conducted at a rate which is sufficient to establish and maintain the desired concentration in the test chamber. The test is started within 30 minutes after the test chemical has been added and uniformly distributed in static test chambers or after the concentration of test chemical in each flow-through test chamber reaches the prescribed level and remains stable. At the initiation of the test, daphnids which have been cultured and acclimated in accordance with the test design are randomly placed into the test chambers. Daphnids in the test chambers are observed periodically during the test, the immobile daphnids removed, and the findings recorded.

(ii) Dissolved oxygen concentration, pH, temperature, the concentration of test chemical and other water quality parameters are measured at specified intervals in selected test chambers. Data are collected during the test to develop concentration-response curves and determine $EC_{50}$ values for the test chemical.

(2) [Reserved]

(3) *Range-finding test.* (i) A range-finding test should be conducted to establish test solution concentrations for the definitive test.

(ii) The daphnids should be exposed to a series of widely spaced concentrations of the test chemical (e.g., 1, 10, 100 mg/1, etc.), usually under static conditions.

(iii) A minimum of five daphnids should be exposed to each concentration of test chemical for a period of 48 hours. The exposure period may be shortened if data suitable for the purpose of the range-finding test can be obtained in less time. No replicates are required and nominal concentrations of the chemical are acceptable.

(4) *Definitive test.* (i) The purpose of the definitive test is to determine the concentration-response curves and the 24- and 48-hour $EC_{50}$ values with the minimum amount of testing beyond the range-finding test.

(ii) A minimum of 20 daphnids per concentration shall be exposed to five or more concentrations of the chemical chosen in a geometric series in which the ratio is between 1.5 and 2.0 (e.g., 2, 4, 8, 16, 32, and 64 mg/l). An equal number of daphnids shall be placed in two or more replicates. If solvents, solubilizing agents or emulsifiers have to be used, they shall be commonly used carriers and shall not possess a synergistic or antagonistic effect on the toxicity of the test chemical. The concentration of solvent should not exceed 0.1 mg/l. The concentration ranges shall be selected to determine the concentration-response curves and $EC_{50}$ values at 24 and 48 hours. Concentration of test chemical in test solutions should be analyzed prior to use.

(iii) Every test shall include controls consisting of the same dilution water, conditions, procedures and daphnids from the same population (culture container), except that none of the chemical is added.

(iv) The dissolved oxygen concentration, temperature and pH shall be measured at the beginning and end of the test in each chamber.

(v) The test duration is 48 hours. The test is unacceptable if more than 10 percent of the control organisms are immobilized during the 48-hour test period. Each test chamber shall be checked for immobilized daphnids at 24 and 48 hours after the beginning of the test. Concentration-response curves and 24-hour and 48-hour $EC_{50}$ values for immobilization shall be determined along with their 95 percent confidence limits.

(vi) In addition to immobility, any abnormal behavior or appearance shall also be reported.

(vii) Test organisms shall be impartially distributed among test chambers in such a manner that test results show no significant bias from the distributions. In addition, test chambers within the testing area shall be positioned in a random manner or in a way in which appropriate statistical analyses can be used to determine the variation due to placement.

(viii) The concentration of the test chemical in the chambers should be measured as often as is feasible during the test. In the static test the concentration of test chemical shall be measured, at a minimum, at the beginning of the test and at the end of the test in each test chamber. In the flow-through test the concentration of test chemical shall be measured at a minimum:

(A) In each chamber at the beginning of the test and at 48 hours after the start of the test;

(B) In at least one appropriate chamber whenever a malfunction is detected in any part of the test substance delivery system.

Among replicate test chambers of a treatment concentration, the measured concentration of the test chemical shall not vary more than ±20 percent.

(5) [Reserved]

(6) *Analytical measurements.* (i) *Test chemical.* Deionized water should be used in making stock solutions of the test chemical. Standard analytical methods should be used whenever available in performing the analyses. The analytical method used to measure the amount of test chemical in a sample shall be validated before beginning the test by appropriate laboratory practices. Any analytical method is not acceptable if likely degradation products of the test chemical, such as hydrolysis and oxidation products, give positive or negative interferences which cannot be systematically identified and corrected mathematically.

(ii) *Numerical.* The number of immobilized daphnids shall be counted during each definitive test. Appropriate statistical analyses should provide a goodness-of-fit determination for the concentration-response curves. A 24- and 48-hour $EC_{50}$ and corresponding 95 percent interval shall be calculated.

(d) *Test conditions*—(1) *Test species*—(i) *Selection.* (A) The cladocerans, *Daphnia magna* or *D. pulex*, are the test species to be used in this test. Either species may be used for testing of a particular chemical. The species identity of the test organisms should be verified using appropriate systematic keys. First instar daphnids, <24 hours old, are to be used to start the test.

(B) Daphnids to be used in acute toxicity tests should be cultured at the test facility. Records should be kept regarding the source of the initial stock and culturing techniques. All organisms used for a particular test shall have originated from the same culture population.

(C) Daphnids shall not be used for a test (*1*) if cultures contain ephippia; (*2*) if adults in the cultures do not produce young before day 12; (*3*) if more than 20 percent of the culture stock die during the 2 days preceding the test; (*4*) if adults in the culture do not produce an average of at least 3 young per adult per day over the 7-day period prior to the test and (*5*) if daphnids have been used in any portion of a previous test, either in a treatment or in a control.

(ii) *Acclimation.* (A) Brood daphnids shall be maintained in 100-percent dilution water at the test temperature for at least 48 hours prior to the start of the test. This is easily accomplished by culturing them in the dilution water at the test temperature. During production of neonates, daphnids should not be fed.

(B) During culturing and acclimation to the dilution water, daphnids should be maintained in facilities with background colors and light intensities similar to those of the testing area.

(iii) *Care and handling.* (A) Daphnids should be cultured in dilution water under similar environmental conditions to those used in the test. Organisms should be handled as little as possible. When handling is necessary it should be done as gently, carefully, and quickly as possible. During culturing and acclimation, daphnids should be observed carefully for ephippia and other signs of stress, physical damage and mortality. Dead and abnormal individuals shall be discarded. Organisms that touch dry surfaces or are dropped or injured in handling shall be discarded.

(B) Smooth glass tubes (I.D. greater than 5 mm) equipped with rubber bulb should be used for transferring daphnids with minimal culture media carry-over. Care should be exercised to introduce the daphnids below the surface of any solution to avoid trapping air under the carapace.

(iv) *Feeding.* A variety of foods (e.g., unicellular green algae) have been demonstrated to be adequate for daphnid culture. Daphnids shall not be fed during testing.

(2) *Facilities*—(i) *Apparatus.* (A) Facilities needed to perform this test include: (*1*) Containers for culturing and acclimating daphnids; (*2*) a mechanism for controlling and maintaining the water temperature during the culturing, acclimation, and test periods; (*3*) apparatus for straining particulate matter, removing gas bubbles, or aerating the water as necessary; and (*4*) an apparatus for providing a 16-hour light and 8-hour dark photoperiod with a 15 to 30 minute transition period. In addition, the flow-through system shall contain appropriate test chambers in which to expose daphnids to the test chemical and an appropriate test substance delivery system.

(B) Facilities should be well ventilated and free of fumes and disturbances that may affect the test organisms.

(C) Test chambers shall be loosely covered to reduce the loss of test solution or dilution water due to evaporation and to minimize the entry of dust or other particulates into the solutions.

(ii) *Construction materials.* (A) Materials and equipment that contact test solutions should be chosen to minimize sorption of test chemicals from the dilution water and should not contain substances that can be leached into aqueous solution in quantities that can affect the test results.

(B) For static tests, daphnids can be conveniently exposed to the test chemical in 250 ml beakers or other suitable containers.

(C) For flow-through tests, daphnids can be exposed in glass or stainless steel containers with stainless steel or nylon screen bottoms. The containers should be suspended in the test chamber in such a manner to insure that the test solution flows regularly into and out of the container and that the daphnids are always submerged in at least 5 centimeters of test solution. Test chambers can be constructed using 250 ml beakers or other suitable containers equipped with screened overflow holes, standpipes or V-shaped notches.

(iii) *Dilution water.* (A) Surface or ground water, reconstituted water or dechlorinated tap water are acceptable as dilution water if daphnids will survive in it for the duration of the culturing, acclimation and testing periods without showing signs of stress. The quality of the dilution water should be constant and should meet the following specifications:

| Substance | Maximum concentration |
|---|---|
| Particulate matter | 20 mg/liter. |
| Total organic carbon or | 2 mg/liter. |
| Chemical oxygen demand | 5 mg/liter. |
| Un-ionized ammonia | 1 µg/liter. |
| Residual chlorine | <3 µg/liter. |
| Total organophosphorus pesticides | 50 ng/liter. |
| Total organochlorine pesticides plus polychlorinated biphenyls (PCBs) or. | 50 ng/liter. |
| Organic chlorine | 25 ng/liter. |

(B) The above water quality parameters under paragraph (d)(2)(iii)(A) of this section shall be measured at least twice a year or whenever it is suspected that these characteristics may have changed significantly. If dechlorinated tap water is used, daily chlorine analysis shall be performed.

(C) If the diluent water is from a ground or surface water source, conductivity and total organic carbon (TOC) or chemical oxygen demand (COD) shall be measured. Reconstituted water can be made by adding specific amounts of reagent-grade chemicals to deionized or distilled water. Glass distilled or carbon-filtered deionized water with a conductivity less than 1 µohm/cm is acceptable as the diluent for making reconstituted water.

(iv) *Cleaning.* All test equipment and test chambers shall be cleaned before each use using standard laboratory procedures.

(v) *Test substance delivery system.* In flow-through tests, proportional diluters, metering pump systems, or other suitable devices should be used to deliver test chemical to the test chambers. The system shall be calibrated before each test. Calibration includes determining the flow rate through each chamber and the concentration of the test chemical in each chamber. The general operation of the test substance delivery system should be checked twice during a test. The 24-hour flow through a test chamber shall be equal to at least 5 times the volume of the test chamber. During a test, the flow rates should not vary more than 10 percent from any one test chamber to another.

(3) *Test parameters.* Environmental parameters of the water contained in test chambers shall be maintained as specified below:

(i) The test temperature shall be 20 °C. Excursions from the test temperature shall be no greater than ±2 °C.

(ii) Dissolved oxygen concentration between 60 and 105 percent saturation. Aeration, if needed to achieve this level, shall be done before the addition of the test chemical. All treatment and control chambers shall be given the same aeration treatment.

(iii) The number of daphnids placed in a test chamber shall not affect test results. Loading shall not exceed 40 daphnids per liter test solution in the static system. In the flow-through test, loading limits will vary depending on the flow rate of dilution water. Loading shall not cause the dissolved oxygen concentration to fall below the recommended levels.

(iv) Photoperiod of 16 hours light and 8 hours darkness.

(e) *Reporting.* The sponsor shall submit to the U.S. EPA all data developed by the test that are suggestive or predictive of acute toxicity and all concomitant gross toxicological manifestations. In addition to the reporting requirements prescribed in Part 792—*Good Laboratory Practice Standards* of this chapter, the reporting of test data shall include the following:

(1) The name of the test, sponsor, testing laboratory, study director, principal investigator, and dates of testing.

(2) A detailed description of the test chemical including its source, lot number, composition (identity and concentration or major ingredients and major impurities), known physical and chemical properties and any carriers or other additives used and their concentrations.

(3) The source of the dilution water, its chemical characteristics (e.g., conductivity, hardness, pH, etc.) and a description of any pretreatment.

(4) Detailed information about the daphnids used as brood stock, including the scientific name and method of verification, age, source, treatments, feeding history, acclimation procedures, and culture method. The age of the daphnids used in the test shall be reported.

(5) A description of the test chambers, the volume of solution in the chambers, the way the test was begun (e.g., conditioning, test chemical additions), the number of test organisms per test chamber, the number of replicates per treatment, the lighting, the method of test chemical introduction or the test substance delivery system and the flow rate (in flow-through test) expressed as volume additions per 24 hours.

(6) The concentration of the test chemical in each test chamber at times designated for static and flow-through tests.

(7) The number and percentage of organisms that were immobilized or showed any adverse effects in each test chamber at each observation period.

(8) Utilizing the average measured test chemical concentration, concentration-response curves should be fitted to immobilization data at 24 and 48 hours. A statistical test of goodness-of-fit should be performed and the results reported.

(9) The 24- and 48-hour $EC_{50}$ values and their respective 95 percent confidence limits using the mean measured test chemical concentration and the methods used to calculate both the $EC_{50}$ values and their confidence limits.

(10) All chemical analyses of water quality and test chemical concentrations, including methods, method validations and reagent blanks.

(11) The data records of the culture, acclimation and test temperatures.

(12) Any deviation from this test guideline and anything unusual about

the test, e.g., diluter failure, temperature fluctuations, etc.

[50 FR 39321, Sept. 27, 1985, as amended at 52 FR 19059, May 20, 1987]

### § 797.1330 Daphnid chronic toxicity test.

(a) *Purpose.* This guideline is intended for use in developing data on the chronic toxicity of chemical substances and mixtures ("chemicals") subject to environmental effects test regulations under the Toxic Substances Control Act (TSCA) (Pub. L. 94-469, 90 Stat. 2003, 15 U.S.C. 2601 *et seq.*). This guideline prescribes a chronic toxicity test in which daphnids are exposed to a chemical in a renewal or a flow-through system. The United States Environmental Protection Agency will use data from this test in assessing the hazard a chemical may present to the aquatic environment.

(b) *Definitions.* The definitions in section 3 of the Toxic Substances Control Act (TSCA), and the definitions in Part 792 *Good Laboratory Practice Standards* of this chapter apply to this test guideline. In addition, the following definitions apply to this guideline:

(1) "Brood stock" means the animals which are cultured to produce test organisms through reproduction.

(2) "Chronic toxicity test" means a method used to determine the concentration of a substance in water that produces an adverse effect on a test organism over an extended period of time. In this test guideline, mortality and reproduction (and optionally, growth) are the criteria of toxicity.

(3) "$EC_{50}$" means that experimentally derived concentration of test substance in dilution water that is calculated to affect 50 percent of a test population during continuous exposure over a specified period of time. In this guideline, the effect measured is immobilization.

(4) "Ephippium" means a resting egg which develops under the carapace in response to stress conditions in daphnids.

(5) "Flow-through" means a continuous or intermittent passage of test solution or dilution water through a test chamber or culture tank with no recycling.

(6) "Immobilization" means the lack of movement by daphnids except for minor activity of the appendages.

(7) "Loading" means the ratio of daphnid biomass (grams, wet weight) to the volume (liters) of test solution in a test chamber at a point in time or passing through the test chamber during a specific interval.

(8) "MATC (Maximum Acceptable Toxicant Concentration)" means the maximum concentration at which a chemical can be present and not be toxic to the test organism.

(9) "Renewal system" means the technique in which test organisms are periodically transferred to fresh test solution of the same composition.

(c) *Test procedures*—(1) *Summary of the test.* (i) Test chambers are filled with appropriate volumes of dilution water. In the flow-through test the flow of dilution water through each chamber is then adjusted to the rate desired. The test substance is introduced into each test chamber. The addition of test substance in the flow-through system is done at a rate which is sufficient to establish and maintain the desired concentration of test substance in the test chamber.

(ii) The test is started within 30 minutes after the test substance has been added and uniformly distributed in the test chambers in the renewal test or after the concentration of test substance in each test chamber of the flow-through test system reaches the prescribed level and remains stable. At the initiation of the test, daphnids which have been cultured or acclimated in accordance with the test design, are randomly placed into the test chambers. Daphnids in the test chambers are observed periodically during the test, immobile adults and offspring produced are counted and removed, and the findings are recorded. Dissolved oxygen concentration, pH, temperature, the concentration of test substance, and other water quality parameters are measured at specified intervals in selected test chambers. Data are collected during the test to determine any significant differences ($p<0.05$) in immobilization and reproduction as compared to the control.

(2) [Reserved]

(3) *Range-finding test.* (i) A range-finding test should be conducted to establish test solution concentrations for the definitive test.

(ii) The daphnids should be exposed to a series of widely spaced concentrations of the test substance (e.g., 1, 10, 100 mg/l), usually under static conditions.

(iii) A minimum of five daphnids should be exposed to each concentration of test substance for a period of time which allows estimation of appropriate chronic test concentrations. No replicates are required and nominal concentrations of the chemical are acceptable.

(4) *Definitive test.* (i) The purpose of the definitive test is to determine concentration-response curves, $EC_{50}$ values and effects of a chemical on immobilization and reproduction during chronic exposure.

(ii) A minimum of 20 daphnids per concentration shall be exposed to five or more concentrations of the chemical chosen in a geometric series in which the ratio is between 1.5 and 2.0 (e.g., 2, 4, 8, 16, 32, 64 mg/l). An equal number of daphnids shall be placed in two or more replicates. The concentration ranges shall be selected to determine the concentration-response curves, $EC_{50}$ values and MATC. Solutions shall be analyzed for chemical concentration at designated times during the test.

(iii) Every test shall include controls consisting of the same dilution water, conditions, procedures and daphnids from the same population (culture container), except that none of the chemical is added.

(iv) The test duration is 21 days. The test is unacceptable if:

(A) More than 20 percent of the control organisms appear to be immobilized, stressed or diseased during the test.

(B) Each control daphnid living the full 21 days produces an average of less than 60 young.

(C) Any ephippia are produced by control animals.

(v) The number of immobilized daphnids in each chamber shall be recorded on day 21 of the test. After offspring are produced, they shall be counted and removed from the test chambers every 2 or 3 days. Concentration-response curves, $EC_{50}$ values and associated 95 percent confidence limits for adult immobilization shall be determined for day 21. An MATC shall be determined for the most sensitive test criteria measured (number of adult animals immobilized, number of young per adult, and number of immobilized young per adult).

(vi) In addition to immobility, any abnormal behavior or appearance shall also be reported.

(vii) Test organisms shall be impartially distributed among test chambers in such a manner that test results show no significant bias from the distributions. In addition, test chambers within the testing area shall be positioned in a random manner as in a way in which appropriate statistical analyses can be used to determine the variation due to placement.

(5) [Reserved]

(6) *Analytical measurements.* (i) *Test chemical.* Deionized water should be used in making stock solutions of the test substance. Standard analytical methods should be used whenever available in performing the analyses. The analytical method used to measure the amount of test substance in a sample shall be validated before beginning the test by appropriate laboratory practices. An analytical method is not acceptable if likely degradation products of the test substance, such as hydrolysis and oxidation products, give positive or negative interferences which cannot be systematically identified and corrected mathematically.

(ii) *Numerical.* The number of immobilized adults, total offspring per adult, and immobilized offspring per adult shall be counted during each test. Appropriate statistical analyses should provide a goodness-of-fit determination for the adult immobilization concentration-response curves calculated on day 21. A 21-day $EC_{50}$ based on adult immobilization and corresponding 95 percent confidence intervals shall also be calculated. Appropriate statistical tests (e.g., analysis of variance, mean separation test) should be used to test for significant chemical effects on chronic test criteria (cumulative number of immobilized adults, cumulative number of offspring per adult and cumulative number of immobilized offspring per adult) on day 21. An MATC shall be calculated using these chronic test criteria.

(d) *Test conditions*—(1) *Test species*—(i) *Selection.* (A) The cladocerans, *Daphnia magna* or *D. pulex*, are the species to be used in this test. Either species can be utilized for testing of a particular chemical. The species identity of the test organisms should be verified using appropriate systematic keys.

(B) First instar daphnids, <24 hours old, are to be used to start the test.

(ii) *Acquisition.* (A) Daphnids to be used in chronic toxicity tests should be cultured at the test facility. Records should be kept regarding the source of the initial stock and culturing techniques. All organisms used for a particular test shall have originated from the same culture population.

(B) Daphnids shall not be used for a test if:

(*1*) Cultures contain ephippia.

(*2*) Adults in the cultures do not produce young before day 12.

(*3*) More than 20 percent of the culture stock die in the 2 days preceding the test.

(*4*) Adults in the culture do not produce an average of at least 3 young per adult per day over the 7-day period prior to the test.

(*5*) Daphnids have been used in any portion of a previous test either in a treatment or in a control.

(iii) *Feeding.* (A) During the test the daphnids shall be fed the same diet and with the same frequency as that used for culturing and acclimation. All treatments and control(s) shall receive, as near as reasonably possible, the same ration of food on a per-animal basis.

(B) The food concentration depends on the type used. Food concentrations should be sufficient to support normal growth and development and to allow for asexual (parthenogenic) reproduction. For automatic feeding devices, a suggested rate is 5 to 7 mg food (either solids or algal cells, dry weight) per liter dilution water or test solution. For manual once-a-day feeding, a suggested rate is 15 mg food (dry weight) per liter dilution water or test solution.

(iv) *Loading.* The number of test organisms placed in a test chamber shall not affect test results. Loading shall not exceed 40 daphnids per liter in the renewal system. In the flow-through test, loading limits will vary depending on the flow rate of the dilution water. Loading shall not cause the dissolved oxygen concentration to fall below the recommended level.

(v) *Care and handling of test organisms.* (A) Daphnids should be cultured in dilution water under similar environmental conditions to those used in the test. A variety of foods have been demonstrated to be adequate for daphnid culture. They include algae, yeasts and a variety of mixtures.

(B) Organisms should be handled as little as possible. When handling is necessary it should be done as gently, carefully, and quickly as possible. During culturing and acclimation, daphnids should be observed carefully for ephippia and other signs of stress, physical damage, and mortality. Dead and abnormal individuals shall be discarded. Organisms that touch dry surfaces or are dropped or injured during handling shall be discarded.

(C) Smooth glass tubes (I.D. greater than 5mm) equipped with a rubber bulb can be used for transferring daphnids with minimal culture media carry-over.

(D) Care should be exercised to introduce the daphnids below the surface of any solution so as not to trap air under the carapace.

(vi) *Acclimation.* (A) Brood daphnids shall be maintained in 100 percent dilution water at the test temperature for at least 48 hours prior to the start of the test. This is easily accomplished by culturing them in dilution water at the test temperature. During acclimation, daphnids shall be fed the same food as will be used for the definitive test.

(B) During culturing and acclimation to the dilution water, daphnids should be maintained in facilities with background colors and light intensities similar to those of the testing area.

(2) *Facilities*—(i) *General.* (A) Facilities needed to perform this test include:

(*1*) Containers for culturing and acclimating daphnids.

(*2*) A mechanism for controlling and maintaining the water temperature during the culturing, acclimation and test periods.

(*3*) Apparatus for straining particulate matter, removing gas bubbles, or aerating the water when water supplies contain particulate matter, gas bubbles, or insufficient dissolved oxygen, respectively.

(*4*) An apparatus for providing a 16-hour light and 8-hour dark photoperiod.

(*5*) An apparatus to introduce food if continuous or intermittent feeding is used.

(*6*) In addition, the flow-through test shall contain appropriate test chambers in which to expose daphnids to the test substance and an appropriate test substance delivery system.

(B) Facilities should be well ventilated and free of fumes and other disturbances that may affect the test organisms.

(ii) *Test chambers.* (A) Materials and equipment that contact test solutions should be chosen to minimize sorption of test chemicals from the dilution water and should not contain substances that can be leached into aqueous solution in quantities that can affect test results.

(B) For renewal tests, daphnids can be conveniently exposed to the test solution in 250 ml beakers or other suitable containers.

(C) For flow-through tests daphnids can be exposed in glass or stainless steel containers with stainless steel or nylon screen bottoms. Such containers shall be suspended in the test chamber in such a manner to ensure that the test solution flows regularly into and out of the container and that the daphnids are always submerged in at least 5 centimeters of test solution. Test chambers can be constructed using 250 ml beakers or other suitable containers equipped with screened overflow holes, standpipes or V-shaped notches.

(D) Test chambers shall be loosely covered to reduce the loss of test solution or dilution water due to evaporation and to minimize the entry of dust or other particulates into the solutions.

(iii) *Test substance delivery system.* (A) In the flow-through test, proportional diluters, metering pump systems or other suitable systems should be used to deliver the test substance to the test chambers.

(B) The test substance delivery system shall be calibrated before each test. Calibration includes determining the flow rate through each chamber and the concentration of the test substance in each chamber. The general operation of the test substance delivery system should be checked twice daily during a test. The 24-hour flow rate through a test chamber shall be equal to at least five times the volume of the test chamber. During a test, the flow rates shall not vary more than 10 percent from any one test chamber to another. For the renewal test, test substance dilution water shall be completely replaced at least once every 3 days.

(iv) *Dilution water.* (A) Surface or ground water, reconstituted water, or dechlorinated tap water are acceptable as dilution water if daphnids will survive in it for the duration of the culturing, acclimation, and testing periods without showing signs of stress. The quality of the dilution water should be constant and should meet the following specificiations:

| Substance | Maximum concentration |
|---|---|
| Particulate matter | 20 mg/l. |
| Total organic carbon or | 2 mg/l. |
| Chemical oxygen demand | 5 mg/l. |
| Un-ionized ammonia | 20 μg/l. |
| Residual chlorine | <3 μg/l. |
| Total organophosphorus pesticides | 50 ng/l. |
| Total organochlorine pesticides plus polychlorinated biphenyls (PCBs). | 50 ng/l. |
| or organic chlorine | 25 ng/l. |

(B) The water quality characteristics listed above shall be measured at least twice a year or when it is suspected that these characteristics may have changed significantly. If dechlorinated tap water is used, daily chlorine analysis shall be performed.

(C) If the diluent water is from a ground or surface water source, conductivity and total organic carbon (TOC) or chemical oxygen demand (COD) shall be measured. Reconstituted water can be made by adding specific amounts of reagent-grade chemicals to deionized or distilled water. Glass distilled or carbon filtered deionized water with a conductivity of less than 1 microohm/cm is acceptable as the diluent for making reconstituted water.

(D) If the test substance is not soluble in water an appropriate carrier should be used.

(v) *Cleaning of test system.* All test equipment and test chambers shall be cleaned before each use following standard laboratory procedures. Cleaning of test chambers may be necessary during the testing period.

(3) *Test parameters.* (i) Environmental conditions of the water contained in test chambers should be maintained as specified in this paragraph:

(A) The test temperature shall be 20 °C. Excursions from the test temperature shall be no greater than ±2 °C.

(B) Dissolved oxygen concentration between 60 and 105 percent saturation. Aeration, if needed to achieve this level, shall be done before the addition of the test substance. All treatment and control chambers shall be given the same aeration treatment.

(C) Photoperiod of 16-hours light and 8-hours darkness.

(ii) Additional measurements include:

(A) The concentration of the test substance in the chambers shall be measured during the test.

(B) At a minimum, the concentration of test substance should be measured as follows:

(*1*) In each chamber before the test.

(*2*) In each chamber on days 7, 14, and 21 of the test.

(*3*) In at least one appropriate chamber whenever a malfunction is detected in any part of the test substance delivery system. Equal aliquots of test solution may be removed from each replicate chamber and pooled for analysis. Among replicate test chambers of a treatment concentration, the measured concentration of the test substance should not vary more than 20 percent.

(*4*) An apparatus for providing a 16-hour light and 8-hour dark photoperiod.

(C) The dissolved oxygen concentration, temperature and pH shall be measured at the beginning of the test and on days 7, 14, and 21 in at least two chambers of the high, middle, low, and control test concentrations.

(e) *Reporting.* The sponsor shall submit to the U.S. Environmental Protection Agency all data developed by the test that are suggestive or predictive of chronic toxicity and all associated toxicologic manifestations. In addition to the reporting requirements prescribed in the Part 792—*Good Laboratory Practice Standards* of this chapter the reporting of test data shall include the following:

(1) The name of the test, sponsor, testing laboratory, study director, principal investigator, and dates of testing.

(2) A detailed description of the test substance including its source, lot number, composition (identity and concentration of major ingredients and major impurities), known physical and chemical properties, and any carriers or other additives used and their concentrations.

(3) The source of the dilution water, its chemical characteristics (e.g., conductivity, hardness, pH), and a description of any pretreatment.

(4) Detailed information about the daphnids used as brood stock, including the scientific name and method of verification, age, source, treatments, feeding history, acclimation procedures, and culture methods. The age of the daphnids used in the test shall be reported.

(5) A description of the test chambers, the volume of solution in the chambers, the way the test was begun (e.g., conditioning, test substance additions), the number of test organisms per test chamber, the number of replicates per treatment, the lighting, the renewal process and schedule for the renewal chronic test, the test substance delivery system and flow rate expressed as volume additions per 24 hours for the flow-through chronic test, and the method of feeding (manual or continuous) and type of food.

(6) The concentration of the test substance in test chambers at times designated for renewal and flow-through tests.

(7) The number and percentage of organisms that show any adverse effect in each test chamber at each observation period.

(8) The cumulative adult and offspring immobilization values and the progeny produced at designated observation times, the time (days) to first brood and the number of offspring per adult in the control replicates and in each treatment replicate.

(9) All chemical analyses of water quality and test substance concentrations, including methods, method validations and reagent blanks.

(10) The data records of the culture, acclimation, and test temperatures.

(11) Any deviation from this test guideline, and anything unusual about the test, (e.g., dilution failure, temperature fluctuations).

(12) The MATC to be reported is calculated as the geometric mean between the lowest measured test substance concentration that had a significant ($p<0.05$) effect and the highest measured test substance concentration that had no significant ($p<0.05$) effect on day 21 of the test. The most sensitive of the test criteria (number of adult animals immobilized, the number of young per female and the number of immobilized young per female) is used to calculate the MATC. The criterion selected for MATC computation is the one which exhibits an effect (a statistically significant difference between treatment and control groups; $p<0.05$) at the lowest test substance concentration for the shortest period of exposure. Appropriate statistical tests (analysis of variance, mean separation test) shall be used to test for significant test substance effects. The statistical tests employed and the results of these tests shall be reported.

(13) Concentration-response curves utilizing the average measured test substance concentration shall be fitted to cumulative adult immobilization data at 21 days. A statistical test of goodness-of-fit shall be performed and the results reported.

(14) An $EC_{50}$ value based on adult immobilization with corresponding 95 percent confidence limits when sufficient data are present for day 21. These calculations shall be made using the average measured concentration of the test substance.

[50 FR 39321, Sept. 27, 1985, as amended at 52 FR 19060, May 20, 1987]

## § 797.1350 Daphnid chronic toxicity test.

(a) *Purpose.* The proposed *Daphnia* chronic toxicity test standard is designed to assess the effects of test substances on the survival and reproduction of Daphnia as a representative freshwater invertebrate. The duration of the test permits the daphnids to be exposed to a chemical from shortly after birth until well into adulthood. The organisms are exposed long enough to allow the adults to produce several broods of progeny. Initiating exposure shortly after birth allows an assessment of the possible effects of the test chemical on such processes as reproduction, maturation, fecundity and growth.

(b) *Definitions.* The following definitions apply to this standard:

(1) "Acute lethal toxicity" is the lethal effect produced on an organism within a short period of time of exposure to a chemical.

(2) "Confidence limits" are the limits within which, at some specified level of probability, the true value of a result lies.

(3) "$LC_{50}$" is the median lethal concentration, i.e., that concentration of a chemical in air or water killing 50 percent of a test batch of organisms within a particular period of exposure (which shall be stated).

(4) "Reference substance" is a chemical used to access the constancy of response of a given species of test organisms to that chemical, usually by use of the acute $LC_{50}$. (It is assumed that any change in sensitivity to the reference substance will indicate the existence of some similar change in degree of sensitivity to other chemicals whose toxicity is to be determined.)

(5) "Static test" is a toxicity test with aquatic organisms in which no flow of test solution occurs. Solutions may remain unchanged throughout the duration of the test.

(6) "Renewal test" is a test without continuous flow of solution, but with occasional renewal of test solutions after prolonged periods, e.g., 24 hours.

(7) "Flow-through test" is a toxicity test in which water is renewed continuously in the test chambers, the test chemical being transported with the water used to renew the test medium.

(8) "Time-response curve" is the curve relating cumulative percentage response of a test batch of organisms, exposed to a single dose or single concentration of a chemical, to a period of exposure.

(9) "Toxicity curve" is the curve produced from toxicity tests when $LC_{50}$ values are plotted against duration of exposure. (This term is also used in aquatic toxicology, but in a less precise sense, to describe the curve produced when the median period of survival is plotted against test concentrations).

(10) "Units" all concentrations are given in weight per volume (e.g., in mg/liter).

(c) *Test procedures*—(1) *Summary of the test.* (i) Test chambers are filled with appropriate volumes of dilution water. In the flow-through test the flow of dilution water through each chamber is then adjusted to the rate desired. The test substance is introduced into each test chamber. The addition of test substance in the flow-through system is done at a rate which is sufficient to establish and maintain the desired concentration of test substance in the test chamber.

(ii) For the renewal test, the test is started within 30 minutes after the test substance has been added and uniformly distributed in the test chambers. In the flow-through test the test begins after the concentration of test substance in each test chamber of the flow-through test system reaches the prescribed level and remains stable. At the initiation of the test, daphnids which have been cultured or acclimated in accordance with the test design, are randomly placed into the test chambers. Daphnids in the test chambers are observed periodically during the test, immobile adults and offspring produced are counted and removed, and the findings are recorded. Dissolved oxygen concentration, pH, temperature, the concentration of te substance, and other water quality parameters are measured at specified intervals in selected test chambers. Dat are collected during the test to determine any significant difference ($p<0.05$) in immobilization and reproduction as compared to the control.

(2) [Reserved]

(3) *Range-finding test*—(i) *Genera* A range-finding test should be conducted to establish test solution concentrations for the definitive test.

(ii) *Introductory information fo range-finding test*—(A) *Prerequisites:*

(*1*) Water solubility.

(*2*) Vapor pressure.

(B) *Guidance information:*

(*1*) Structural formula.

(*2*) Purity of the substance.

(*3*) Methods of analysis for th quantification of the substance i water.

(*4*) Chemical stability in water an light.

(*5*) *n*-octanol/water partition coefficient.

(*6*) Results of a test on biodegradability.

(C) *Qualifying statement.* Fo chemicals with low solubility unde test conditions, it may not be possibl to quantitatively determine the $EC_{50}$.

(iii) *Methods for range-finding test*—(A) *Definitions and units:* (*1*) *24 hou $EC_{50}$*. The concentration (based upo nominal concentration) calculated t have immobilized 50 percent of th daphnids by 24 hours exposure. (If another definition is used, this shall b reported, together with its reference.)

(*2*) *Immobilization.* Those animal not able to swim for 15 seconds afte gentle agitation of the test containe are considered to be immobile. (If another definition is used, this shall b reported, together with its reference.)

(B) *Reference substances.* In th course of the acute immobilizatio phase a reference substance may occasionally be tested for $EC_{50}$ with th test compound as a means of assurin that the laboratory test conditions ar adequate and have not changed significantly. An example of such useful reference substance is $K_2CR_2O_7$.

(C) *Conditions for the validity of th range-finding test:* (*1*) The mortalit in the controls should not exceed 10 percent at the end of the test.

(*2*) The oxygen concentration at the end of the test shall be >70 percent of the air saturation value at the temperature used.

(*3*) Test Daphnia should not have been trapped at the surface of the water, at least in the control.

(*4*) If conducted, the results with the reference compound should be within the normal range for the laboratory conducting the test.

(*5*) If the $EC_{50}$ is not calculable due to an inadequate number of intermediate response levels, it is acceptable to merely report the highest concentration causing complete immobility, provided that the concentration factor between doses was <1.8.

(D) *Performance of the range-finding test:* (*1*) Equipment which will come into contact with the test solutions should be glass. This glassware should be cleaned with solvents known to remove previously tested chemicals.

(*2*) Any water, either reconstituted or natural water, can be used, provided that it will sustain growth and reproduction of *Daphnia* without signs of stress.

(*3*) At least 20 animals should be used at each test concentration, preferably divided into four batches of five animals each.

(*4*) At least 2 ml of test solution should be provided for each animal.

(*5*) The test temperature should be between 18 and 22 °C, and for each test it should be constant within ±0.5 °C.

(*6*) A light-dark cycle is optional.

(*7*) The concentrations should be formulated in a geometric series, preferably without using any solvents. If solvents, solubilizing agents, emulsifiers, etc., have to be used, they should be commonly used adjuvants and not be toxic in themselves at the levels used. Neither should they have a synergistic or antagonistic effect on the toxicity of the substance tested. In no case should the concentration of an organic solvent exceed 0.1 ml/l.

(*8*) The test solution should be prepared before introduction of the daphnids.

(*9*) The test solutions should not be aerated.

(*10*) The daphnids shall not be fed during the test.

(*11*) The highest concentration to be tested should not exceed 1.0 g/l.

(*12*) Concentrations sufficient to lead to zero and 100 percent immobilization and the 24 hour $EC_{50}$ should be tested together with a control.

(*13*) The pH and the oxygen concentration of the blank and all the test concentrations should be measured at the beginning and the end of the test. The pH of the test solutions should not be modified.

(*14*) Volatile compounds should be tested in completely filled, closed containers, large enough to prevent lack of oxygen.

(4) *Definitive test*—(i) *General.* The results of the range-finding test are used to determine, with judgment, the concentration levels to be used in the definitive test. It is suggested that this reproduction test be carried out using a geometrical concentration series of at least 5 concentrations with an interval of at least 10, starting at approximately the 24 hour $EC_{50}$ concentration and ending at $\frac{1}{100}$ of the 24 hour $EC_{50}$. If necessary, lower concentrations are to be tested.

(ii) *Introductory information for the definitive test*—(A) *Prerequisities:*

(*1*) Water solubility.

(*2*) Vapor pressure.

(*3*) Chemical stability in water and light.

(*4*) Results of a test on biodegradability.

(*5*) 24 hour $EC_{50}$ or the highest concentration producing no immobility and the lowest concentration causing complete immobility.

(B) *Guidance information:* (*1*) Structural formula.

(*2*) Purity of the substance.

(*3*) *n*-octanol/water partition coefficient.

(C) *Recommendations.* (*1*) Instead of a 2-week test in which 3 batches of young should be born per female, a test of 3 or 4 weeks may be preferred in order to obtain a more thorough judgment of the influence of the test substance on mortality and reproduction. In this period approximately six to nine batches of young should be born per female.

(*2*) It is recommended that a statistical test (such as an analysis of variance) be used to determine whether the test replications can be analyzed together.

(iii) *Criteria for a valid definitive test.* (A) Control mortality should not exceed 20 percent at the end of the test.

(B) The oxygen concentration shall have been >70 percent of the air saturation value throughout the test.

(C) The pH for the controls and for at least the most concentrated solutions shall be known throughout the test. The deviation from the initial value at the beginning of the test should be <0.3 units.

(D) The first young should have been born in the controls after a maximum of 9 days.

(E) The average cumulative number of young per female in the controls after three broods, should be >20 at a temperature of 20° ± 0.5°C.

(iv) *Definitive test procedures.* (A) At least 40 animals should be used at each test concentration, preferably divided into 4 batches of 10 animals each. The test concentrations are made up in a geometric series, and if possible, without any solvents.

(B) Every test shall include controls consisting of the same dilution water, conditions, procedures and daphnids from the same population (culture container), except that none of the chemical is added.

(C) The minimum duration of the test is 14 days, in which at least three broods of the F1 generation shall have appeared in the controls. If this is not the case, the test shall be continued until the third brood in the control is complete. If desired, the test can be continued for a total period of 3 to 4 weeks, even if three broods are born within 3 weeks.

(D) The live and dead daphnids of the "parental" generation (P) are counted and the dead specimens removed. This should preferably be carried out daily, but at least every 2 days, e.g., Monday, Wednesday, and Friday.

(E) The presence of eggs in the brood pouch, males or epphipia shall be recorded. The condition and size of the parent generation should be visually compared with the controls.

(F) When the parental animals are about 7 days old, the first young daphnids emerge from the brood pouch. After this, a new batch appears every 2 to 3 days. These batches are called "broods" of the F1 generation.

(G) The newborn young of the F1 generation are counted at least every 2 days (Monday, Wednesday, and Friday) and their estimated condition (based on visual examination) is recorded. After counting and examination, the young are poured away. The presence of eggs from which no young have emerged (on the bottom of the test vessel) is recorded.

(5) [Reserved]

(6) *Analytical measurements.* (i) Deionized water should be used in making stock solutions of the test substance. Standard analytical methods should be used whenever available in performing the analyses. The analytical method used to measure the amount of test substance in a sample shall be validated before beginning the test by appropriate laboratory practices. An analytical method should not be used if likely degradation products of the test substance, such as hydrolysis and oxidation products, give positive or negative interferences which cannot be systematically identified and corrected mathematically.

(ii) Samples of the test substance should be taken at the beginning and during the test. The actual concentration shall not drop below 80 percent of the nominal concentration. Aeration of the test solutions is permissible, unless this would cause the actual concentration of the test substance to drop below 80 percent of the nominal concentration.

(iii) The oxygen concentration in all test solutions shall be checked once every 48 hours (if desired, every Monday, Wednesday and Friday).

(iv) The pH of the controls and of at least the most concentrated solution shall be checked before and after each renewal in the renewal test and once every 48 hours in a flow-through test.

(d) *Test conditions*—(1) *Test species*—(i) *Selection. Daphnia magna* less than 24 hours old at the beginning of the test, laboratory bred, free from known diseases and with a known history (breeding method, pretreatment) are used in this test. Other *Daphnia* species may be used provided that the relevant re-production parameters are comparable to those of *Daphnia magna*.

(ii) *Feeding.* The daphnids should be fed at least daily during the definitive test. In the chronic daphnid test, food (in any quantity) of any kind that meets the criteria of reproduction for validity of the test, is acceptable. Overloading of the test system with food should be avoided in order to minimize sorption of the test substance. Log-phase, unicellular green algae are generally suitable.

(2) *Facilities*—(i) *Apparatus.* (A) Normal laboratory apparatus and equipment should be used. Equipment which will come into contact with the test solutions should preferably be all glass. This glassware should be cleaned with solvents known to remove previously tested chemicals.

(B) This reproduction test should not be carried out in a static test system; either a renewal or flow-through system shall be used. The renewal period should be guided by the chemical analysis and (if applicable) the oxygen level in the test solution. The solutions shall be renewed at least once every 48 hours (if desired, on Monday, Wednesday, and Friday).

(C) Volatile compounds should be tested in completely filled closed containers, large enough to prevent the oxygen concentrations from falling below 70 percent of the saturation value. An almost-closed, flow-through system may also be used. When more than 20 percent of the test compound would be lost through volatility, the test should be carried out either in a flow-through system or in an enclosed container of sufficient size to ensure that the oxygen level does not fall below 70 percent of the saturation value.

(ii) *Cleaning.* If the renewal scheme is used, the glassware shall be emptied and food residues removed at renewal. It is recommended that the glassware be rinsed with deionized water and kept as a coded series for the following renewal. Each test unit therefore has two vessels which are used alternately. If flow-through systems are used, these should be cleaned twice a week.

(iii) *Dilution water.* Surface or ground water, reconstituted water, or dechlorinated tap water is acceptable as dilution water if daphnids will survive and reproduce in it for the duration of the culturing, acclimation, and testing periods without showing signs of stress. The quality of the dilution water should be constant and should meet the following specifications:

| Substance | Maximum concentration |
|---|---|
| Particulate matter | 20 mg/l. |
| Total organic carbon or | 2 mg/l. |
| chemical oxygen demand | 5 mg/l. |
| Un-ionized ammonia | 20 μg/l. |
| Residual chlorine | 1 μg/l. |
| Total organophosphorus pesticides. | 50 ng/l. |
| Total organochlorine pesticides plus polychlorinated biphenyls (PCBs). | 50 ng/l. |
| or organic chlorine | 25 ng/l. |

(3) *Test parameters*—(i) *Carriers.* If solvents, solubilizing agents, emulsifiers, etc., have to be used, they should be commonly used adjuvants and should not themselves be toxic at the concentrations used. They should also not interact to alter the toxicity of the substance under test. In no case should the concentration of an organic solvent exceed 0.1 ml/l.

(ii) *Dissolved oxygen.* The oxygen concentration shall be >70 percent of the air saturation value throughout the test.

(iii) *Lighting.* A light-dark cycle is necessary for the definitive test; 8 hours darkness and 16 hours light are recommended.

(iv) *Loading.* At least 40 ml of test solution should be provided for each animal in the definitive test.

(v) *Temperature.* The test temperature should be between 18 and 22 °C, but for each test it should be constant, within ±0.5 °C.

(e) *Reporting*—(1)(i) *Test substance information:* (A) Chemical designation.

(B) Additional designations, e.g., trade name.

(C) Empirical formula.

(D) Manufacturer.

(E) Batch number.

(F) Degree of purity.

(G) Date of sampling.

(H) Water solubility.

(I) Vapor pressure.

(J) Biodegradability.

(K) Chemical stability in water and daylight.

(L) *n*-octanol/water partition coefficient.

(ii) *General information:* (A) Source of Daphnia, any pretreatment, breeding method (including source, kind and amount of food, feeding frequency), species identification and method of verification.

(B) Name and address of the testing laboratory, name of the person responsible for carrying out the test (study director).

(C) Name and address of sponsor.

(D) Dates of testing.

(E) Description of the test method or reference to the method used.

(iii) *Conditions of testing:* (A) Carriers and/or additives used and their concentrations. If it is observed that the stability or homogeneity of the test substance cannot be maintained, then care should be taken in the interpretation of the results and note made that these may not be reproducible.

(B) Dilution water: source and chemical and physical characteristics including at least hardness, pH, Ca/Mg ratio, Na/K ratio, alkalinity.

(C) Test temperature.

(D) Light quality, intensity, and periodicity.

(E) All measurements of pH and oxygen level made during the test, preferably in tabular form.

(F) Results and date of test performed with reference compound if available.

(G) Description of test vessels: volume of solution, number of test organisms per vessel, number of test vessels per concentration, conditioning of the test vessels, the introduction of the test substance in the dilution water.

(H) In case of renewal, the renewal procedure and scheme. In case of flow-through, the test substance delivery system, the flowrate, periodicity of cleaning and technique used.

(I) If measured, the actual concentrations of the test substance and the dates of measurement.

(J) Number and percentage of daphnids that showed any adverse effect in the controls and in each treatment at each observation period and a description of the nature of the effects observed, e.g., immobilization, mortality in tabular form.

(K) Description or reference to statistical procedures applied.

(L) Any other effects differentiating organisms in tests and controls.

(iv) *Specific range-finding and definitive test information:* (A) For the 24-hour EC$_{50}$ (acute immobilization) phase also report:

(*1*) The 24-hour EC$_{50}$ preferably with 95 percent confidence limits, either by computation or graphically, and the method applied. The probit method is recommended.

(*2*) If possible, the slope of the concentration response curve with its 95 percent confidence limits.

(*3*) The highest tested concentration producing no immobile daphnids.

(*4*) The lowest tested concentration producing 100 percent immobile daphnids.

(*5*) Any other effect observed and the concentration at which it occurred.

(B) For the reproduction phase also report:

(*1*) The EC$_{50}$ (immobilization) and LC$_{50}$ values as far as possible at 24 hours, 48 hours, 96 hours, 7 days, 14 days and at the end of the test, preferably with 95 percent confidence limits, either by computation or graphically, and the method applied. For the determination, a probit method should be used.

(*2*) The length of time for the appearance of the first brood for each concentration.

(*3*) The number of young alive in each test vessel on given days when counts were made (the minimum requirement is for counts at 48-hour intervals on Mondays, Wednesdays, and Fridays).

(*4*) The number of dead young in each test vessel on given days when counts were made.

(*5*) Source, kind and amount of food; feeding frequency.

(*6*) If the recommended concentration scheme was followed and no effects on reproduction are detected, then the results may be reported as being greater than the highest concentration tested.

(*7*) For each of the observed effects a statistical analysis of the homogeneity of replicate results for each concentration should be made. If homogeneity is found, it should be determined through an appropriate statistical analysis, whether a significant difference exists between the control and the test concentrations.

(*8*) The highest concentration tested at which no significant difference is found compared to the controls with respect to mortality, reproduction and other observed effects.

(*9*) The lowest concentration tested with significant difference compared to the controls.

(*10*) Any other parameter can be reported at the option of the study director.

(2) [Reserved]

## § 797.1400 Fish acute toxicity test.

(a) *Purpose.* This guideline may be used to develop data on the acute toxicity of chemical substances and mixtures ("chemicals") subject to environmental effects test regulations under the Toxic Substances Control Act (TSCA) (Pub. L. 94-469, 90 Stat. 2003, 15 U.S.C. 2601 *et seq.*). This guideline prescribes tests to be used to develop data on the acute toxicity of chemicals to fish. The United States Environmental Protection Agency (EPA) will use data from these tests in assessing the hazard of a chemical to the environment.

(b) *Definitions.* The definitions in section 3 of the Toxic Substances Control Act (TSCA), and the definitions in Part 792—*Good Laboratory Practice Standards* of this chapter apply to this test guideline. The following definitions also apply to this guideline:

(1) "Acclimation" means the physiological compensation by test organisms to new environmental conditions (e.g., temperature, hardness, pH).

(2) "Acute toxicity test" means a method used to determine the concentration of a substance that produces a toxic effect on a specified percentage of test organisms in a short period of time (e.g., 96 hours). In this guideline, death is used as the measure of toxicity.

(3) "Carrier" means a solvent used to dissolve a test substance prior to delivery to the test chamber.

(4) "Conditioning" means the exposure of construction materials, test chambers, and testing apparatus to dilution water or to test solutions prior to the start of a test in order to minimize the sorption of the test substance onto the test facilities or the leaching of substances from the test facilities into the dilution water or test solution.

(5) "Death" means the lack of opercular movement by a test fish.

(6) "Flow-through" means a continuous or an intermittent passage of test solution or dilution water through a test chamber, or a holding or acclimation tank with no recycling.

(7) "Incipient $LC_{50}$" means that test substance concentration, calculated from experimentally-derived mortality data, that is lethal to 50 percent of a test population when exposure to the test substance is continued until the mean increase in mortality does not exceed 10 percent in any concentration over a 24-hour period.

(8) "$LC_{50}$" means that test substance concentration, calculated from experimentally-derived mortality data, that is lethal to 50 percent of a test population during continuous exposure over a specified period of time.

(9) "Loading" means the ratio of fish biomass (grams, wet weight) to the volume (liters) of test solution in a test chamber or passing through it in a 24 hour period.

(10) "Static" means the test solution is not renewed during the period of the test.

(11) "Test solution" means the test substance and the dilution water in which the test substance is dissolved or suspended.

(c) *Test procedures*—(1) *Summary of the test.* (i) Test chambers are filled with appropriate volumes of dilution water. If a flow-through test is performed, the flow of dilution water through each chamber is adjusted to the rate desired.

(ii) The test substance is introduced into each test chamber. In a flow-through test, the amount of test substance which is added to the dilution water is adjusted to establish and maintain the desired concentration of test substance in each test chamber.

(iii) Test fish which have been acclimated in accordance with the test design are introduced into the test and control chambers by stratified random assignment.

(iv) Fish in the test and control chambers are observed periodically during the test; dead fish are removed at least twice each day and the findings are recorded.

(v) The dissolved oxygen concentration, pH, temperature and the concentration of test substance are measured at intervals in selected test chambers.

(vi) Concentration-response curves and $LC_{50}$ values for the test substance are developed from the mortality data collected during the test.

(2) [Reserved]

(3) *Range finding test.* If the toxicity of the test substance is not already known, a range finding test should be performed to determine the range of concentrations to be used in the definitive test. The highest concentration of test substance for use in the range finding test should not exceed its solubility in water or the permissible amount of the carrier used.

(4) *Definitive test.* (i) A minimum of 20 fish should be exposed to each of five or more test substance concentrations. The range of concentrations to which the fish are exposed should be such that in 96 hours there are at least two partial mortality exposures bracketing 50 percent survival.

(ii) For exposure to each concentration of a test substance, an equal number of test fish shall be placed in two or more replicate test chambers. Test fish shall be impartially distributed among test chambers in such a manner that test results show no significant bias from the distributions.

(iii) Every test shall include a control consisting of the same dilution water, conditions, procedures, and fish from the same group used in the test, except that none of the test substance is added.

(iv) Mortality data collected during the test are used to calculate a 96-hour $LC_{50}$. The 24-, 48-, and 72-hour values should be calculated whenever there is sufficient mortality data to determine such values. If the 96-hour $LC_{50}$ is less than 50 percent of the estimated 48-hour $LC_{50}$ in a flow-through test, the test shall be continued until the mean increase in mortality at any test concentration does not exceed 10 percent over a 24-hour period or until 14 days.

(v) Test fish shall not be fed while they are being exposed to the test substance under static conditions or during the first 96 hours of flow-through testing. If the test continues past 96 hours, the fish should be fed a suitable food at a maintenance level every other day beginning on test day 5. Any excess food and the fecal material should be removed when observed.

(5) *Test results.* (i) Death is the primary criterion used in this test guideline to evaluate the toxicity of the test substance.

(ii) In addition to death, any abnormal behavior such as, but not limited to, erratic swimming, loss of reflex, increased excitability, lethargy, or any changes in appearance or physiology such as discoloration, excessive mucous production, hyperventilation, opaque eyes, curved spine, or hemorrhaging shall be recorded.

(iii) Observations on compound solubility shall be recorded. The investigator shall report the appearance of surface slicks, precipitates, or material adhering to the sides of the test chamber.

(iv) Each test and control chamber shall be checked for dead fish and observations recorded at 24, 48, 72, and 96 hours after the beginning of the test or within one hour of the designated times. If the test is continued past 96 hours, additional observations shall be made every 24 hours until termination.

(v) The mortality data is used to calculate $LC_{50}$'s and their 95 percent confidence limits, and to plot concentration-response curves for each time interval whenever sufficient data exists. The methods recommended for use in calculating $LC_{50}$'s include probit, logit, binomial, and moving average angle.

(vi) A test is unacceptable if more than 10 percent of the control fish die or exhibit abnormal behavior during a 96-hour test. If a flow-through test is continued past 96 hours, the maximum allowable additional mortality is 10 percent.

(6) *Analytical measurements*—(i) *Water quality analysis.* (A) The hardness, acidity, alkalinity, pH, conductivity, TOC or COD, and particulate matter of the dilution water should be measured at the beginning of each static test and at the beginning and end of each flow-through test. The month to month variation of the above values should be less than 10 percent and the pH should vary less than 0.4 units.

(B) During static tests, the dissolved oxygen concentration, temperature, and pH shall be measured in each test chamber at the beginning and end of the test. The test solution volume shall not be reduced by more than 10 percent as a result of these measurements.

(C) During flow-through tests, dissolved oxygen, temperature and pH measurements shall be made in each chamber at the beginning and end of the test.

(ii) *Collection of samples for measurement of test substance.* Test solution samples to be analyzed for the test substance should be taken midway between the top, bottom, and sides of the test chamber. These samples should not include any surface scum or material dislodged from the bottom or sides. Samples should be analyzed immediately or handled and stored in a manner which minimizes loss of test substance through microbial degradation, photodegradation, chemical reaction, volatilization, or sorption.

(iii) *Measurement of test substance.* (A) For static tests, the concentration of the test substance shall be measured at a minimum in each test chamber at each test concentration at the beginning (0-hour, before fish are added) and at the end of the test. During flow-through tests, the concentration of test substance shall be measured as follows:

(*1*) In at least the chamber of each test concentration at 0-hour.

(*2*) In at least the chamber of each test concentration at 96-hours and every 4 days thereafter, as long as the test is continued.

(*3*) In at least one appropriate chamber whenever a malfunction is detected in any part of the test substance delivery system.

(*4*) Equal aliquots of test solution may be removed from each replicate chamber and pooled for analysis.

(B) Filters and their holders used for determining the dissolved test substance concentrations should be prewashed with several volumes of distilled water and undergo a final rinse with test solution. Glass or stainless steel filter holders are best for organic test substances, while plastic holders are best for metals. The sample should be filtered within 30 minutes after it is taken from the test chamber.

(C) The analytical methods used to measure the amount of test substance in a sample shall be validated before beginning the test. The accuracy of a method should be verified by a method such as using known additions. This involves adding a known amount of the test substance to three water samples taken from a chamber containing dilution water and the same number and species of fish as are used in the test. The nominal concentration of the test substance in those samples should span the concentration range to be used in the test.

(D) An analytical method is not acceptable if likely degradation products of the test substance give positive or negative interferences, unless it is shown that such degradation products are not present in the test chambers during the test.

(E) In addition to analyzing samples of test solution, at least one reagent blank, containing all reagents used, should also be analyzed.

(F) If the measured concentrations of dissolved test substance are considerably lower (e.g., <50 percent) than the nominal concentrations, the total test substance concentration should be measured in the highest test concentration.

(G) Among replicate test chambers, the measured concentrations shall not vary more than 20 percent. The measured concentration of the test substance in any chamber during the test should not vary more than 30 percent from the measured concentration at time 0.

(H) The mean measured concentration of test substance shall be used to calculate all $LC_{50}$'s and to plot all concentration-response curves.

(d) *Test conditions*—(1) *Test species*—(i) *Selection.* The test species for this test are the rainbow trout (*Salmo gairdneri*), bluegill (*Lepomis macrochirus*) and fathead minnow (*Pimephales promelas*). The particular species of fish to be used will be prescribed in the test rule.

(ii) *Age and condition of fish.* (A) Juvenile fish shall be used. Fish used in a particular test shall be the same age and be of normal size and appearance for their age. The longest fish shall not be more then twice the length of the shortest.

(B) All newly acquired fish should be quarantined and observed for at least 14 days prior to use in a test.

(C) Fish shall not be used for a test if they appear stressed or if more than five percent die during the 48 hours immediately prior to the test.

(iii) *Acclimation of test fish.* (A) If the holding water is not from the same source as the test dilution water, acclimation to the dilution water should be done gradually over a 48-hour period. The fish should then be held an additional 14 days in the dilution water prior to testing. Any changes in water temperature should not exceed 3 °C per day. Fish should be held for a minimum of 7 days at the test temperature prior to testing.

(B) During the final 48-hours of acclimation, fish should be maintained in facilities with background colors and light intensities similar to those of the testing area and should not be fed.

(2) *Facilities*—(i) *General.* Facilities needed to perform this test include:

(A) Flow-through tanks for holding and acclimating fish.

(B) A mechanism for controlling and maintaining the water temperature during the holding, acclimation and test periods.

(C) Apparatus for straining particulate matter, removing gas bubbles, or insufficient dissolved oxygen, respectively.

(D) Apparatus for providing a 16-hour light and 8-hour dark photoperiod with a 15- to 30-minute transition period.

(E) Chambers for exposing test fish to the test substance.

(F) A test substance delivery system for flow-through tests.

(ii) *Construction materials.* Construction materials and commercially purchased equipment that may contact the stock solution, test solution, or dilution water should not contain substances that can be leached or dissolved into aqueous solutions in quantities that can alter the test results. Materials and equipment that contact stock or test solutions should be chosen to minimize sorption of test chemicals. Glass, stainless steel, and perfluorocarbon plastic should be used whenever possible. Concrete, fiberglass, or plastic (e.g., PVC) may be used for holding tanks, acclimation tanks, and water supply systems, but they should be used to remove rust particles. Rubber, copper, brass, galvanized metal, epoxy glues, and lead should not come in contact with the dilution water, stock solution, or test solution.

(iii) *Test substance delivery system.* In flow-through tests, diluters, metering pump systems, or other suitable devices should be used to deliver the test substance to the test chambers. The system used should be calibrated before each test. Calibration includes determining the flow rate through each chamber and the concentration of the test substance delivered to each chamber. The general operation of the test substance delivery system should be checked twice daily during a test. The 24-hour flow rate through a test chamber should be a minimum of 6 tank volumes. During a test, the flow rates should not vary more than 10 percent from one test chamber to another.

(iv) *Test chambers.* Test chambers made of stainless steel should be welded, not soldered. Test chambers made of glass should be fused or bonded using clear silicone adhesive. As little adhesive as possible should be left exposed in the interior of the chamber.

(v) *Cleaning of test system.* Test substance delivery systems and test chambers should be cleaned before each test. They should be washed with detergent and then rinsed in sequence with clean water, pesticide-free acetone, clean water, and 5 percent nitric acid, followed by two or more changes of dilution water.

(vi) *Dilution water.* (A) Clean surface or ground water reconstituted water, or dechlorinated tap water is acceptable as dilution water if the test fish will survive in it for the duration of the holding, acclimating, and testing periods without showing signs of stress, such as discoloration, hemorrhaging, disorientation or other unusual behavior. The quality of the dilution water should be constant and should meet the following specifications measured at least twice a year:

| Substance | Maximum |
|---|---|
| Particulate matter | 20 mg/liter. |
| Total organic carbon or | 2 mg/liter. |
| chemical oxygen demand | 5 mg/liter. |
| Un-ionized ammonia | 1 µg/liter. |
| Residual chlorine | 1 µg/liter. |
| Total organochloring pesticides | 50 µg/liter. |
| Total organocholorine pesticides plus polychlorinated biphenyls (PCBs). | 50 µg/liter. |
| or organic chlorine | 25 µg/liter. |

(B) The concentration of dissolved oxygen in the dilution water should be between 90 and 100 percent saturation; 9.8 to 10.9 mg/l for tests with trout, and 8.0 to 8.9 mg/l for tests with bluegill or fathead minnow at sea level. If necessary, the dilution water can be aerated before the addition of the test substance. All reconstituted water should be aerated before use. Buffered soft water should be aerated before but not after the addition of buffers.

(C) If disease organisms are present in the dilution water in sufficient numbers to cause infection, they should be killed or removed by suitable equipment.

(D) Glass distilled or carbon filtered deionized water with a conductivity less than 1 micromho/cm is acceptable for use in making reconstituted water. If the reconstituted water is prepared from a ground or surface water source, conductivity, and total organic carbon (TOC) or chemical oxygen demand (COD) should be measured on each batch.

(vii) *Carriers.* (A) Distilled water should be used in making stock solutions of the test substance. If the stock volume however is more than 10 percent of the test solution volume, dilution water should be used. If a carrier is absolutely necessary to dissolve the test substance, the volume used should not exceed the minimum volume necessary to dissolve or suspend the test substance in the test solution. If the test substance is a mixture, formulation, or commercial product, none of the ingredients is considered a carrier unless an extra amount is used to prepare the stock solution.

(B) Triethylene glycol and dimethyl formamide are the preferred carriers, but acetone can also be used. The concentration of triethylene glycol in the test solution should not exceed 80 mg/l. The concentration of dimethyl formamide or acetone in the test solution should not exceed 5/0 mg/l.

(3) *Test parameters*—(i) *Loading.* The number of fish placed in a test chamber should not be so great as to affect the results of the test. The loading should not be so great that the test substance concentrations are decreased by more than 20 percent due to uptake by the fish. In static tests, loading should not exceed 0.5 grams of fish per liter of solution in the test chamber at any one time. In flow-through tests loading should not exceed 0.5 grams of fish per liter of test solution passing through the chamber in 24 hours. These loading rates should be sufficient to maintain the dissolved oxygen concentration above the recommended levels and the ammonia concentration below 20 µg/l.

(ii) *Dissolved oxygen concentration.* (A) During static tests with rainbow trout the dissolved oxygen in each test chamber shall be greater than 5.5 mg/l. In tests with bluegill and fathead minnows, the DO shall be maintained above 4.5 mg/l.

(B) During flow-through tests the dissolved oxygen concentration shall be maintained above 8.2 mg/l in tests with trout and above 6.6 mg/l in tests with bluegills or fathead minnows.

(iii) *Temperature.* The test temperature shall be 22 °C for rainbow trout. Excursions from the test temperature shall be no greater than ±2 °C. The temperature shall be measured at least hourly in one test chamber.

(iv) *Light.* A 16-hour light and 8-hour dark photoperiod should be maintained.

(e) *Reporting.* The sponsor shall submit to the EPA all data developed by the test that are suggestive or predictive of toxicity. In addition to the reporting requirements prescribed in Part 792—*Good Laboratory Practice Standards* of this chapter, the reported test data shall include the following:

(1) The source of the dilution water, a description of any pretreatment, and the measured hardness, acidity, alkalinity, pH, conductivity, TOC or COD and particulate matter.

(2) A description of the test chambers, the depth and volume of solution in the chamber, the specific way the test was begun (e.g., conditioning, test substance additions), and for flow-through tests, a description of the test substance delivery system.

(3) Detailed information about the test fish, including the scientific name and method of verification, average weight (grams, wet weight), standard length, age, source, history, observed diseases, treatments, and mortalities, acclimation procedures, and food used.

(4) The number of replicates used, the number of organisms per replicate, the loading rate, and the flow rate for flow-through tests.

(5) The measured DO, pH and temperature and the lighting regime.

(6) The solvent used, the test substance concentration in the stock solution, the highest solvent concentration in the test solution and a description of the solubility determinations in water and solvents if used.

(7) The concentrations of the test substance at each test concentration just before the start of the test and at all subsequent sampling periods.

(8) The number of dead and live tests organisms, the percentage of organisms that died, and the number that showed any abnormal effects in the control and in each test chamber at each observation period.

(9) The 96-hour $LC_{50}$, and when sufficient data have been generated, the 24-, 48-, 72-, and incipient $LC_{50}$ values, their 95 percent confidence limits, and the methods used to calculate the $LC_{50}$ values and their confidence limits.

(10) When observed, the observed no effect concentration (the highest concentration tested at which there were no mortalities or abnormal behavioral or physiological effects).

(11) The concentration-response curve at each observation period for which a $LC_{50}$ was calculated.

(12) Methods and data records of all chemical analyses of water quality parameters and test substance concentrations, including method validations and reagent blanks.

[50 FR 39321, Sept. 27, 1985, as amended at 52 FR 19062, May 20, 1987]

## § 797.1440 Fish acute toxicity test.

(a) *Purpose.* This guideline will be used in developing data on the acute toxicity of chemical substances and mixtures ("chemicals") to fish subject to environmental effects test regulations under the Toxic Substances Control Act (TSCA) (Pub. L. 94-469, 90 Stat. 2003, 15 U.S.C. 2601 *et seq.*). The United States Environmental Protection Agency (EPA) will use data from these tests in assessing the hazard of a chemical to the environment.

(b) *Definitions.* The definitions in section 3 of the Toxic Substances Control Act (TSCA) and in Part 792—*Good Laboratory Practice Standards* of this chapter apply to this test guideline. The following definitions also apply:

(1) "Acute toxicity" is the discernible adverse effects induced in an organism within a short period of time (days) of exposure to a chemical. For aquatic animals this usually refers to continuous exposure to the chemical in water for a period of up to four days. The effects (lethal or sub-lethal) occurring may usually be observed within the period of exposure with aquatic organisms.

(2) "Acute lethal toxicity" is the lethal effect produced on an organism within a short period of time of exposure to a chemical.

(3) "Confidence limits" are the limits within which, at some specified level of probability, the true value of a result lies.

(4) "$LC_{50}$" is the median lethal concentration, i.e., that concentration of a chemical in air or water killing 50 percent of a test batch of organisms within a particular period of exposure (which should be stated).

(5) "Static test" is a toxicity test with aquatic organisms in which no flow of test solution occurs. (Solutions may remain unchanged throughout the duration of the test).

(6) "Semi-static test" is a test without flow of solution, but with occasional batchwise renewal of test solutions after prolonged periods (e.g., 24 hours).

(7) "Flow-through test" is a toxicity test in which water is renewed constantly in the test chambers, the chemical under test being transported with the water used to renew the test medium.

(8) "Time-response curve" is the curve relating cumulative percentage response of a test batch of organisms, exposed to a single dose or single concentration of a chemical, to a period of exposure.

(9) "Toxicity curve" is the curve produced from toxicity tests when $LC_{50}$ values are plotted against duration of exposure. (This term is also used in aquatic toxicology, but in a less precise sense, to describe the curve produced when the median period of survival is plotted against test concentrations.)

(10) "Units" all concentrations are given in weight per volume (e.g., mg/liter).

(c) *Test procedures*—(1) *Summary of the test.* (i) The aqueous solubility and the vapor pressure of the test chemical should be known prior to testing. The structural formula of the test chemical, its purity, stability in water and light, *n*-octanol/water partition coeffecient, and $pK_a$ value should be known. The results of a biodegradability test and the method of analysis for the quantification of the chemical in water should also be known.

(ii) The fish are exposed to a range of test substance concentrations preferably for a period of up to 96 hours. Mortalities are recorded at 24, 48, 72 and 96 hours and the concentrations which kill 50 percent of the fish ($LC_{50}$) are determined where possible.

(iii) The maximum concentration tested producing no mortality and the minimum concentration tested producing total mortality should be recorded.

(iv) For chemicals with limited solubility under the test conditions, it may not be possible to determine an $LC_{50}$.

(2) [Reserved]

(3) *Range-finding test.* It may be necessary to perform a range-finding test prior to a definitive test. It provides information about the range of concentrations to be used in the definitive test.

(4) *Definitive test.* (i) Fish should be exposed to at least five concentrations spaced by a constant factor not exceeding 1.8. A control and solvent control, when appropriate should also be tested.

(ii) Stock solutions of the required strength are prepared by dissolving the appropriate amount of the test substance in the required volume of dilution water. The pH value of the stock solution should be adjusted to the pH value of the dilution water unless there are specific reasons not to do so. The test should be carried out without adjustment of pH if there is evidence of marked change in the pH of the solution, and it is advised that the test be repeated with pH adjustment and the results reported. This pH adjustment should be made in such a way that the stock solution concentration is not changed to any significant extent and that no chemical reaction or physical precipitation of the test compound is caused. NCl or NaOH should be used to adjust the pH.

(iii) Stock solutions of substances of low aqueous solubility may be prepared by ultrasonic dispersion or, if necessary, by use of organic solvents, emulsifiers or dispersants of low toxicity to fish. When such auxiliary substances are used, the control fish should be exposed to the same concentration of the auxiliary substance as that used in the highest concentration of the test substance. The concentration of such auxiliaries should not exceed 0.1 ml/l.

(iv) The chosen test concentrations are prepared by dilution of the stock solution.

(v) For test to be valid, the following criteria apply:

(A) If it is observed that the stability or homogeneity of the test substance cannot be maintained, then care should be taken in the interpretation of the results and a note made that these results may not be reproducible.

(B) The mortality in the controls should not exceed 10 percent at the end of the test.

(C) The dissolved oxygen concentration should have been >60 percent of air saturation throughout the test.

(D) There should be evidence that the concentration of the substance being tested has been satisfactorily maintained (e.g., within 80 percent of the nominal concentration) over the test period.

(5) *Test results.* (i) The fish are inspected after 24, 48, 72, and 96 hours. Fish are considered dead if touching of the caudal peduncle produces no reaction. Dead fish are removed when observed, and mortalities are recorded. Observations after the first 3 hours and 6 hours are desirable.

(ii) Records are kept of visible abnormalities (e.g., loss of equilibrium, swimming behavior, respiratory function, pigmentation, etc.).

(iii) The cumulative percentage mortality for each recommended exposure period should be plotted against concentration on logarithmic-probability paper. A line is then fitted by eye to these points and the concentration corresponding to the 50 percent response point is read off. This is the LC$_{50}$ for the appropriate exposure period. Median lethal concentrations also can be calculated using standard procedures given in any of the references cited in paragraph (f) of this section. Confidence limits (p=0.95) for the calculated LC$_{50}$ values can be determined using the standard procedures. The LC$_{50}$ value should be rounded off to two significant figures.

(iv) Where the data obtained are inadequate for the use of standard methods of calculating the LC$_{50}$ (because most of the results are for either no deaths or total mortality, where a dilution ratio of 1.8 has been used) then the highest concentration causing no deaths and the lowest concentration producing 100 percent deaths should be used to determine the LC$_{50}$ (this being taken as being the geometric mean of these two concentrations).

(6) [Reserved]

(d) *Test conditions*—(1) *Test species*—(i) *Selection.* (A) One of several species may be used, the selection being at the discretion of the testing laboratory. It is suggested that the species used be selected on the basis of such important practical criteria as: their ready availability throughout the year, their ease of maintenance, their convenience for testing, and any economic, biological or ecological factors which have bearing. The fish should be in good health and free from any apparent malformation. If other species fulfilling the above criteria are used, the test method should be adapted in such a way as to provide suitable test conditions.

(B) Examples of fish recommended for testing and their size are given in the following Table 1:

TABLE 1—RECOMMENDED SPECIES FOR ACUTE TESTING

| Recommended species | Recommended total length (centimeter) |
|---|---|
| *Brachydanio rerio* (Teleostei, Cyprinidae) (Hamilton-Buchanan) Zebra-fish | 2.0±1.0 |
| *Pimephales promelas* (Teleostei, Cyprinidae) Fathead minnow | 2.0±1.0 |
| *Cyprinus carpio* (Teleostei, Cyprinidae) (Linne 1758) Common carp | 3.0±1.0 |
| *Oryzias latipes* (Teleostei, Poeciliidae) (Schlegel 1850) Red killifish | 2.0±1.0 |
| *Poecilia reticulata* (Teleostei, Poeciliidae) (Peters 1859) Guppy | 2.0±1.0 |
| *Lepomis macrochirus* (Teleostei, Centrarchidae) (Linneaus 1758) Bluegill | 2.0±1.0 |
| *Salmo gairdneri* (Teleostei, Salmonidae) (Richardson 1836) Rainbow trout | 5.0±1.0 |

(ii) *Collection or acquisition.* The fish mentioned above are easy to rear or are widely available throughout the year. They are capable of being bred and cultivated either in fish farms or in the laboratory under disease- and parasite-controlled conditions so that the test animal will be healthy and of known parentage.

(iii) *Holding and acclimation.* (A) Fish should be held for at least 12 to 15 days before testing. All fish should be maintained in water of the quality to be used in the test for at least 7 days before they are used.

(B) Coldwater fish should be held in tanks containing at least 300 l of water while warmwater fish should be held in tanks containing at least 100 l.

(C) The temperature of the holding water should be the same as that used for testing. The dissolved oxygen concentrations should be maintained above 80 percent of the air saturation value. A 12 to 16 hour photoperiod should be used.

(D) All fish should be fed three times per week or daily until 24 hours before the test is started.

(E) A batch of fish is acceptable for testing if the percentage mortality over the 7-day period prior to testing is less than five. If the mortality is between 5 and 10 percent acclimation should continue for seven 7 days. If the mortality is greater than 10 percent, the entire batch of fish should be rejected.

(2) *Test facilities*—(i) *Apparatus*. An oxygen meter, equipment for determination of water hardness, adequate apparatus for temperature control, test tanks made of chemically inert materials and other normal laboratory equipment are needed.

(ii) *Dilution water*. (A) Drinking water (dechlorinated if necessary), good quality natural water, or reconstituted water, with a total hardness of between 50 and 250 mg/l (as $CaCO)_3$ and with a pH of 6.0–8.5 are preferred.

(B) Reconstituted water should be prepared from deionized water or distilled water with a conductivity <10 $Scm^{-1}$. One hundred liters of reconstituted water can be prepared by adding 2.5 l of the following solutions to a tank and bringing the solution to volume with deionized water:

11.76g $CACl_2 \cdot 2H_2O$/l
4.93g $MgSO_4 \cdot 7H_2O$/l
2.59g $NaHCO_3$/l
2.59g KCl/l

The sum of the calcium and magnesium ions in this solution is 2.5 mmol/l. The proportion of Ca:Mg-ions is 4.13 and of Na:K-ions is 10:1. The acid capacity of this solution is 0.8 mmol/l.

(C) The dilution water should be aerated until oxygen saturation is achieved and then stored for about 2 days without further aeration before use.

(3) *Test parameters*. (i) Constant conditions should be maintained as far as possible throughout the test and, if necessary, semi-static or flow-through procedures should be used.

(ii) The preparation and storage of the test material, the holding of the fish, and all operations and tests should be carried out in an environment free from harmful concentrations of dust, vapors, and gases and in such a way as to avoid cross-contamination. Any disturbances that may change the behavior of the fish should be avoided.

(iii) The following parameters are important:

(A) *Dissolved oxygen*. The dissolved oxygen concentrations should be at least 60 percent of the air saturation value.

(B) *Light*. A 12 to 16 hour photoperiod should be used.

(C) *Loading*. A maximum loading of 1.0 g/l for static and semi-static tests is recommended; for flow-through systems a higher loading can be acceptable.

(D) *Temperature*. Test temperatures of 15 ± 2 °C for rainbow trout and 22 ± 2 °C for carp are recommended. The other recommended species should be tested at 23 ± 2 °C. The temperature should be maintained within ±1 °C of the selected test temperature throughout the test period.

(E) *Feeding*. The fish should not be fed during the test.

(e) *Reporting*. (1) The sponsor should submit to the EPA all data developed by the test that are suggestive or predictive of toxicity.

(2) In addition to the reporting requirements prescribed in Part 792—Good Laboratory Practice Standards of this chapter the reported test data should include the following:

(i) Details of the test procedures used (e.g., static, semi-static, flow-through, aerated, etc.).

(ii) Information about the test organism (scientific name, strain, supplier, any pretreatment, etc.).

(iii) The concentrations tested.

(iv) The number of fish in each test chamber and the loading rate.

(v) The methods of preparation of stock and test solutions.

(vi) The dissolved oxygen concentrations, pH values, temperature, total hardness of the test solutions measured each 24 hours and any other available information on water quality.

(vii) Any available information on the concentrations of the test chemical in the test solutions.

(viii) The maximum concentration causing no mortality within the period of the test.

(ix) The minimum concentration causing 100 percent mortality within the period of the test.

(x) The cumulative mortality in each concentration according to the recommended observation times.

(xi) The $LC_{50}$ values (based on nominal concentrations) at each of the recommended observation times (with 95 percent confidence limits, if possible).

(xii) A graph of the concentration-mortality curve at the end of the test.

(xiii) The statistical procedures used for determining the $LC_{50}$ values.

(xiv) The mortality of the control animals.

(xv) Any incidents in the course of the test which might have influenced the results.

(xvi) Any abnormal responses of the fish.

(xvii) A statement that the test was carried out in agreement with the prescriptions of the Test Guideline given above (otherwise a description of any deviations occurring).

(f) *References.* For additional background information on this test guideline the following references should be consulted:

(1) American Public Health Association, *Standard methods for the examination of water and wastewater.* 14th ed. (American Public Health Association: New York, 1975).

(2) U.S. Environmental Protection Agency, Committee on Methods for Toxicity Tests with Aquatic Organisms, "methods for acute toxicity tests with fish, macroinvertebrates and amphibians," EPA Report No. 660/3-75-009 (Corvallis, Oregon, 1975).

(3) Finney, A.J. *Statistical Methods in Biological Assay.* (Griffin Ltd.: Weycombe, U.K., 1978).

(4) Litchfield, J.T., Wilcoxon, F. "A simplified method of evaluating dose-effect experiments," *Journal of Pharmacology and Experimental Therapeutics,* 96:99-1113 (1947).

(5) U.S. Environmental Protection Agency, "Methods for measuring the acute toxicity of effluents to aquatic organisms," EPA Report No. 600/4-78-012 (W. Peltier: Cincinnati, Ohio, 1978).

(6) Sprague, J.B. "Measurement of pollutant toxicity to fish. I: Bioassay Methods for Acute Toxicity," *Water Research* 3:794-821 (1969).

(7) ASTM STP 634, Stephan, C.E. Methods for calculating an $LC_{50}$. Eds. Mayer, F.L., Hamelink, J.L. *Aquatic Toxicology and Hazard Evaluation.* American Society for Testing and Materials. (1977) pp.65-84.

(8) Tabata, K. "Quality control of Japanese rice fish for TLm-test;" *Water and Effluent,* 14:1297-1303 (1972).

### § 797.1520 Fish bioconcentration test.

(a) *Purpose.* This guideline is intended to be used for assessing the propensity of chemical substances to bioconcentrate in freshwater fish. This guideline describes a bioconcentration test procedure for the continuous exposure of fathead minnows (*Pimephales promelas*) to a test substance in a flow-through system. The United States Environmental Protection Agency (EPA) will use data from this test in assessing the hazard a chemical may present to the environment.

(b) *Definitions.* The definitions in section 3 of the Toxic Substances Control Act (TSCA) and the definitions in Part 792—*Good Laboratory Practice Standards* of this chapter, are applicable to this test guideline. The following definitions also apply:

(1) "Acclimation" is the physiological compensation by test organisms to new environmental conditions (e.g. temperature, hardness, pH).

(2) "Bioconcentration" is the net accumulation of a substance directly from water into and onto aquatic organisms.

(3) "Bioconcentration factor (BCF)" is the quotient of the concentration of a test substance in aquatic organisms at or over a discrete time period of exposure divided by the concentration in the test water at or during the same time period.

(4) "Carrier" is a solvent used to dissolve a test substance prior to delivery of the test substance to the test chamber.

(5) "Depuration" is the elimination of a test substance from a test organism.

(6) "Depuration phase" is the portion of a bioconcentration test after the uptake phase during which the organisms are in flowing water to which no test substance is added.

(7) "Dilution water" is the water to which the test substance is added and in which the organisms undergo exposure.

(8) "Loading" is the ratio of fish biomass (grams, wet weight) to the volume (liters) of test solution passing through the test chamber during a 24-hr. period.

(9) "Organic chlorine" is the chlorine associated with all chlorine-containing compounds that elute just before lindane to just after mirex during gas chromatographic analysis using a halogen detector.

(10) "Organochlorine pesticides" are those pesticides which contain carbon and chlorine such as aldrin, DDD, DDE, DDT, dieldrin, endrin, and heptachlor.

(11) "Steady-state" is the time period during which the amounts of test substance being taken up and depurated by the test organisms are equal, i.e., equilibrium.

(12) "Steady-state bioconcentration factor" is the mean concentration of the test substance in test organisms during steady-state divided by the mean concentration in the test solution during the same period.

(13) "Stock solution" is the concentrated solution of the test substance which is dissolved and introduced into the dilution water.

(14) "Test chamber" is the container in which the test organisms are maintained during the test period.

(15) "Test solution" is dilution water containing the dissolved test substance to which test organisms are exposed.

(16) "Uptake" is the sorption of a test substance into and onto aquatic organisms during exposure.

(17) "Uptake phase" is the initial portion of a bioconcentration test during which the organisms are exposed to the test solution.

(c) *Test procedures*—(1) *Summary of the test.* (i) Fathead minnows are continuously exposed to at least one constant sublethal concentration of a test substance under flow-through conditions for a maximum of 28 days. During this time, test solution and fish are periodically sampled and analyzed using appropriate methods to quantify the test substance concentration. If prior to day 28, the tissue concentrations of the substance sampled over three consecutive sampling periods have been shown to be statistically similar (i.e., steady-state has been reached), the uptake phase of the test may be terminated and the remaining fish transferred to untreated flowing water until 95 percent of the accumulated residues have been eliminated, or for a maximum depuration period of 14 days.

(ii) The mean test substance concentration in the fish at steady-state is divided by the mean test solution concentration at the same time to estimate the bioconcentration factor (BCF).

(iii) If steady-state is not reached during 28 days of uptake, the steady-state BCF is calculated using nonlinear parameter estimation methods.

(2)—(3) [Reserved]

(4) *Definitive test*—(i) *Background information.* The following data on the test substance shall be known prior to testing:

(A) Its solubility in water.

(B) Its stability in water.

(C) Its octanol-water partition coefficient.

(D) Its acute toxicity to fathead minnows.

(E) The validity accuracy and minimum detection limits of the proposed analytical methods.

(ii) *Selection of test concentration.* (A) At least one concentration shall be tested to assess the propensity of the compound to bioconcentrate. The concentration selected should not stress or adversely affect the fish and should be less than one-tenth the 96-hour or incipient $LC_{50}$ determined from a flow-through test with fathead minnows. The test concentration shall be less than the solubility limit of the compound in water. The test concentration should be close to the potential or expected environmental concentration. The limiting factor of how low one can test is based on the detection limit of the analytical methods. The concentration of the test material in the test solution should be at least 3 times greater than the detection limit in water.

(B) If it is desired to document that the potential to bioconcentrate is independent of the test concentration, at least 2 concentrations shall be tested that are at least a factor of 10 apart.

(iii) *Estimation of test duration.* (A) An estimate of the length of the uptake and depuration phases should be made prior to testing. This will allow the most effective sampling schedule to be determined. The uptake phase shall continue until steady-state has been reached, but need not be longer than 28 days. The test shall continue for at least 4 days.

(B)(*1*) The time to steady state (S in hours) can be estimated from the water solubility or the octanol-water partition coefficient using the following equations:

$S=3.0/\text{antilog }(0.431 \log W-2.11)$ or,
$S=3.0/\text{antilog }(-0.414 \log P+0.122)$
where
$W$=water solubility (mg/l) and
$P$=octanol-water partition coefficient

(*2*) Based upon the estimate of the time to steady state, one of the following sampling schemes may be used to generate the appropriate data.

TIME TO STEADY-STATE IN DAYS

| Test period | S<4 | S>4<14 | S>15<21 | S>21 |
|---|---|---|---|---|
| Sampling Days | | | | |
| Exposure | [1] 1 | [1] 4 | 1 | 1 |
| | [1] 6 | 1 | 3 | 3 |
| | 1 | 3 | 7 | 7 |
| | 2 | 7 | 10 | 10 |
| | 3 | 10 | 14 | 14 |
| | 4 | 12 | 18 | 21 |
| | | 14 | 22 | 28 |
| Depuration | [1] 1 | 1 | 1 | 1 |
| | [1] 6 | 2 | 3 | 3 |
| | [1] 12 | 4 | 7 | 7 |
| | 1 | 6 | 10 | 14 |
| | | | | 14 |

[1] =hours.

(C) The depuration phase shall continue until at least 95 percent of the accumulated test substance and metabolites have been eliminated, but no longer than 14 days.

(iv) *Test initiation.* (A) The test shall not be started until the test substance delivery system has been observed to be functioning properly for at least 48 hours. This time should be sufficient to allow the test substance concentration to become equilibrated with the test exposure system. Analyses of two sets of test solution samples taken prior to test initiation should document this equilibrium (i.e., the concentrations do not vary more than 20 percent from each other). At initiation (time 0), test solution samples shall be collected immediately prior to the addition of fish to the test chambers.

(B) The appropriate number of fathead minnows should be impartially distributed to each test chamber up to five at a time until the appropriate numbers have been distributed. The exact number of test organisms depends upon the expected length of testing, sample size, and the number of additional specialized analyses to be performed at termination.

(v) *Feeding.* (A) Fish shall be fed once a day throughout the uptake and depuration phases. Feeding shall be done just after sampling to minimize the effects of the test substance present in the gut when sampling. Fish should be fed the same food at a similar quantity as they received during holding and acclimation.

(B) Uneaten food and fecal material shall be removed from the test aquaria within 30 minutes after feeding to minimize uptake of test substance by the food or feces.

(vi) *Observations.* (A) Observations on fish appearance and behavior shall be made and recorded daily. Any abnormal behavior such as erratic swimming, lethargy, increased excitability, or any changes in appearances or physiology such as discoloration, hyperventilation or opaque eyes shall be recorded.

(B) Observations on compound solubility shall also be recorded. These include the appearance of surface slicks, precipitates, or material adsorbing to the test chamber.

(vii) *Water quality measurements.* The water temperature and dissolved oxygen concentration shall be recorded at least daily and the pH twice weekly in each test chamber during uptake and depuration.

(viii) *Sampling procedures.* (A) At each of the designated sampling times, triplicate water samples and enough fish should be collected from the exposure chamber(s) to allow for at least four fish tissue analyses. A similar number of control fish should also be collected at each sample point, but only fish collected at the first sampling period and weekly thereafter should be analyzed. Triplicate control water samples will be collected at the time of test initiation and weekly thereafter. Test solution samples should be removed from the approximate center of the water column.

(B) At each sampling period, the appropriate number of fish is netted and removed from each test chamber. Care should be taken not to sample the weakest and consequently usually the smallest fish, especially during the first few sampling periods, to prevent biasing the test results. Each fish is pithed, blotted dry and then frozen at <−10 °C if not analyzed within 4 hours.

(C) At termination, an extra set of fish shall be sampled and eviscerated for quantifying the residues in the viscera and carcass. If a radiolabelled test compound is used, a sufficient number of fish shall be sampled at termination to permit identification and quantitation of any major (>10 percent of parent) metabolites present. It is crucial to determine how much of the activity present in the fish is directly attributable to the parent compound.

(5) *Test results*—(i) *Biological.* (A) The maximum allowable mortality of fish is 10 percent per week. If more than 10 percent of the fish in the control or test chamber(s) die during any week of testing, the test shall be repeated.

(B) Steady-state has been reached when the mean concentrations of test substance in whole fish tissue taken on three consecutive sampling periods are statistically similar (F test, P=0.05). A BCF is then calculated by dividing the mean tissue residue concentration during steady-state by the mean test solution concentration during this same period. A 95 percent confidence interval shall also be derived for the BCF. This can be done by calculating the mean fish tissue concentration at steady state ($X_f$) and its 97.5 percent confidence interval, ±t (S.E.), where t is the t statistic=0.025 and S.E. is one standard error of the mean. This calculation would yield lower and upper confidence limits ($L_f$ and $U_f$). The same procedure can be used to calculate the mean and 97.5 percent confidence interval from the test solution concentrations at steady-state, $X_s$±t (S.E.), and the resulting upper and lower confidence limits ($L_s$ and $U_s$). The 95 percent confidence interval of the BCF would then be between $L_f/U_s$ and $U_f/L_s$.

(C) If steady-state was not reached during the 28-day uptake period, the maximum BCF shall be calculated using the mean tissue concentration from that day and the mean water concentration from that and the previous sampling day. An uptake rate constant shall then be calculated using appropriate techniques, such as the BIOFAC program developed by Blau and Agin (1978) under paragraph (f)(1) of this section. This rate constant will allow the estimation of a steady state BCF and the estimated time to steady-state.

(D) If 95 percent elimination has not been observed after 14 days depuration, then a depuration rate constant shall be calculated. This rate constant will allow estimation of the time to 95 percent elimination.

(ii) *Analytical.* (A) All samples shall be analyzed using EPA methods and guidelines whenever feasible. The specific methodology used shall be validated before the test is initiated. The accuracy of the method should be measured by the method of known additions. This involves adding a known amount of the test substance to three water samples taken from an aquarium containing dilution water and a number of fish equal to that to be used in the test. The nominal concentration of these samples should be the same as the concentration to be used in the test. Samples taken on 2 separate days should be analyzed. The accuracy and precision of the analytical method should be checked using reference or split samples or suitable corroborative methods of analysis. The accuracy of standard solutions should be checked against other standard solutions whenever possible.

(B) An analytical method is not acceptable if likely degradation products of the test substance, such as hydrolysis and oxidation products, give positive or negative interferences, unless it is shown that such degradation products are not present in the test chambers during the test. Atomic absorption spectrophotometric methods for metals and gas chromatographic methods for organic compounds are preferable to colorimetric methods.

(C) In addition to analyzing samples of test solution, at least one reagent blank shall also be analyzed when a reagent is used in the analysis.

(D) When radiolabelled test compounds are used, total radioactivity shall be measured in all samples. At the end of the uptake phase, water and tissue samples should be analyzed using appropriate methodology to identify and estimate the amount of any major (>10 percent of the parent compound) degradation products or metabolites that may be present.

(6) [Reserved]

(d) *Test conditions*—(1) *Test species*—(i) *Selection.* (A) The fathead minnow (*Pimephales promelas*) shall be used as the test organism.

(B) Immature fish should be used. They shall be young enough so as not to mature during the test. Fish used in the same test should be as similar in size as possible to reduce variability. The standard deviation of the weight should be less than 20 percent of the mean (N=30).

(C) Fish used in the same test shall be from the same supplier or culture unit and from the same holding and acclimation tank(s).

(D) Fathead minnows shall not be used if they appear diseased or otherwise stressed or if more than 5 percent die during the 48 hours prior to testing. Diseased fish shall be discarded or treated and held for a minimum of 14 days before testing.

(ii) *Care and handling.* (A) Fish purchased from a commercial source should be attended to immediately upon arrival. Transfer of the fish from the shipping to the holding water should be gradual to reduce stress caused by differences in water quality characteristics and temperature. Fish should be quarantined and observed for at least 14 days prior to testing.

(B) During holding, the fish should not be crowded and the dissolved oxygen concentration shall be above 60 percent saturation. Holding tanks should be kept clean and free of debris. Fish should be fed at least once a day with a food which will support their survival and growth.

(C) Fish shall be handled as little as possible. When handling is necessary, it should be done as gently, carefully, and quickly as possible using dip nets made of small mesh nylon, silk, bolting cloth, plankton netting, or other similar knotless materials. Handling equipment should be sterilized between uses by autoclaving, treating with an iodophor or with 200 mg hypochlorite/liter.

(iii) *Acclimation.* If the holding water is not from the same source as the test dilution water, acclimation to the dilution water should be done over a period of 2 or more days. The fish should then be held an additional 14 days in the dilution water prior to testing. Any changes in water temperature should not exceed 3 °C per day over 72 hours. Fish shall be held for at least 2 days at the test temperature prior to testing.

(iv) *Loading.* The number of fish placed in each test chamber and the flow rate through the test chamber shall be such that the uptake of the test substance by fish upon introduction into the test solution does not reduce the measured concentration of the test solution by more than 20 percent of the concentration measured before the fish were introduced. The loading shall not exceed 0.1 g fish per liter of test solution delivered over any 24-hour period, and the minimum turnover rate shall be 6 aquaria volumes per 24 hours. For some compounds, loading rates less than 0.1g/l may be needed to prevent a substantial loss of test substance as a result of fish uptake.

(2) *Facilities*—(i) *Dilution water.* (A) A constant supply of good quality water shall be available throughout the holding, acclimation and testing periods. Although unadulterated well water is recommended, dechlorinated tap water or reconstituted soft water may be used. A dilution water is acceptable if fathead minnows will survive and grow normally for 60 days without exhibiting signs of stress, i.e., discoloration, lack of feeding, poor response to external stimuli, or lethargy.

(B) The total hardness, alkalinity, pH, specific conductance, temperature and dissolved oxygen concentration of the dilution water shall be determined weekly. The pH should not vary more than 0.4 unit and the other parameters more than 10 percent on a monthly basis.

(C) Reconstituted soft water, if used, should be prepared by adding 4.8 g $NaHCO_3$, 3.0 g $CaSO_4$ $2H_2O$, 3.0 g $MgSO_4$ and 200 mg KCl to each 100 L of deionized or glass-distilled water, or to dechlorinated tap water with a total residual chlorine concentration less than 1 μg/l. In all cases the specific conductance at 25 °C of the water source shall be less than 1 micromho/cm.

(D) All water should be extensively aerated prior to use if the dissolved oxygen concentration is less than 90 percent of saturation. If the concentration of dissolved gases exceeds 110 percent of saturation, the excess gases should be removed using appropriate apparatus.

(E) The quality of the dilution water should be consistent and should meet the following specifications measured at least twice a year.

| Substance | Maximum concentration |
|---|---|
| Particulate matter | 20 mg/liter. |
| Total organic carbon | |
| or | 2 mg/liter. |
| Chemical oxygen demand | 5 mg/liter. |
| Un-ionized ammonia | 1 μg/liter. |
| Residual chlorine | 1 μg/liter. |
| Total organophosphorus pesticides | 50 ng/liter. |
| Total organochlorine pesticides plus polychlorinated biphenyls (PCBs). | |
| or | 50 ng/liter. |
| Organic chlorine | 25 ng/liter. |
| Copper, cadmium or zinc | 10 μg/liter. |

(ii) *Construction materials.* Materials and equipment that contact dilution water, stock solutions or test solutions should not leach or absorb substances. Glass, stainless steel and perfluorocarbon plastics should be used whenever possible. Concrete, unplasticized plastics and fiberglass may be used for holding and acclimation tanks and in the water supply system, but they should be thoroughly conditioned before use by rinsing with a continuous flow of water >25 °C for 48 hours. The use of flexible tubing should be avoided as phthalate esters leach from these materials. Cast iron pipe may be used but filters will be needed to remove rust particles. Rubber, copper, brass, galvanized metal, and epoxy glue should not come in contact with dilution water, stock solutions, or test solutions.

(iii) *Fish holding and acclimation.* (A) Tanks are needed for holding and acclimating fathead minnows prior to testing. The number and size of tanks needed depends upon the amount of testing to be performed and the availability of fish of the right age. A constant supply of good quality dilution water should be supplied to all tanks. The volume required depends upon the holding temperature and the number of fish being held, but the flow should be great enough to maintain a dissolved oxygen concentration >60 percent of saturation.

(B) Temperature control apparatus are needed to maintain the desired holding and acclimation temperatures. Apparatus controls should be able to maintain temperatures within 1 °C of the appropriate temperature. If the water is heated, care should be taken to avoid supersaturation of gases in the water.

(iv) *Testing apparatus.* (A) Test chambers can be made from welded stainless steel or from double strength glass joined with clear silicone adhesive. The size, shape and depth of the test chambers are not important as long as they accommodate the loading requirements.

(B) The test substance delivery system used should accommodate the physical and chemical properties of the test substance and the selected exposure concentration. The apparatus used should accurately and precisely deliver the appropriate amount of stock solution and dilution water to the test chambers. The introduction of the test substance should be done in such a way as to maximize the homogeneous distribution of the test substance throughout the test chamber.

(C) The dilution water should be delivered to an elevated headbox from which it can flow by gravity to the test substance delivery system. Use of a headbox facilitates a constant delivery rate and heating or cooling of the water to the approximate test temperature prior to delivery. Water in the headbox may also be easily aerated or degassed as the situation dictates.

(v) *Cleaning of test apparatus.* Delivery systems and test chambers should be cleaned before and after each use. If there is obvious absorption of a test substance by the silicone adhesive, those applicable parts of the delivery system should be discarded.

(3) *Test parameters*—(i) *Dissolved oxygen.* The dissolved oxygen concentration in each chamber shall be greater than 5.3 mg/l (60 percent of sea-level saturation at 22 °C) throughout testing.

(ii) *Temperature.* The test temperature shall be 22 °C. Excursions from the test temperature shall be no greater than ± 2 °C.

(iii) *Lighting.* A photoperiod of 12 hours light and 12 hours dark with a 15 to 30 minute transition period is recommended.

(iv) *Test substance.* The name and purity of the test substance to be tested will be specified in the test rule. Radio-labelled compounds should not be used unless there are no suitable, validated, analytical techniques to measure unlabelled test substance in fish, or the costs of these analytical techniques are very high.

(v) *Carrier use.* Whenever possible, the test substance should be added directly to the dilution water or from a water stock solution. With compounds having a low water solubility, it may be necessary to prepare test solutions using a carrier. The carriers to be used, in order of preference are: triethylene glycol (TEG), dimethyl formamide (DMF) and acetone. The amount used should be kept to a minimum and should not exceed 80 mg/l in the test solution for TEG and 5.0 mg/l for DMF and acetone.

(e) *Reporting.* In addition to the information required in Part 792—*Good Laboratory Practice Standards* of this chapter, the report shall contain the following:

(1) The source of the dilution water, its mean monthly chemical characteristics (total hardness, alkalinity, pH, specific conductance, temperature and D.O.) and a description of any pretreatment.

(2) Detailed information about the fathead minnows used, including age, mean and standard deviation wet weight (blotted dry) and standard length, source, history of disease, parasites and treatment, acclimation procedures, and food used.

(3) The number of organisms tested, loading rate and volume additions per 24 hours.

(4) The percentage mortality of control fish and fish in each exposure chamber and any observed abnormal behavioral or physiological effects.

(5) The method of stock solution preparation including nominal and measured concentrations and solvent used.

(6) The mean, standard deviation and range of the temperature, dissolved oxygen concentration and pH during the test period.

(7) Photoperiod length and light intensity.

(8) Description of sampling and analytical methods for water and tissue analyses.

(9) The mean, standard deviation and range of the concentration of test compound in the test solution and fish tissue at each sampling period.

(10) The time to steady-state.

(11) The steady-state or maximum BCF and the 95 percent confidence limits.

(12) The time to 95 percent elimination of accumulated residues.

(f) *References.* For further background information on this test guideline the following references should be consulted:

(1) Blau, G.E. and Agin, C.L., "A users manual for BIOFAC: A computer program for characterizing the ratio of uptake and clearance of

chemicals in aquatic organisms," (1978). *Dow Chemical Co.*, Mammalian Environmental Toxicology, Bldg. 1702, Midland, MI 48640.

(2) [Reserved]

[50 FR 39321, Sept. 27, 1985, as amended at 52 FR 19063, May 20, 1987]

### § 797.1560 Fish bioconcentration test.

(a) *Purpose.* This guideline is to be used for assessing the propensity of chemical substances to bioconcentrate in fish. This guideline describes a bioconcentration test procedure for the continuous exposure of fish to a test substance in a flow-through system. The United States Environmental Protection Agency (EPA) will use data from this test in assessing the hazard a chemical may present to the environment.

(b) *Definitions.* The definitions in section 3 of the Toxic Substances Control Act (TSCA) and in Part 792—Good Laboratory Practice Standards of this chapter are applicable to this test guideline. The following definitions also apply:

(1) "Bioconcentration" is the increase in concentration of test material in or on test organisms (or specified tissues thereof) relative to the concentration of test material in the ambient water.

(2) "Bioconcentration factor (BCF)" is the ratio of the test substance concentration in the test fish ($C_f$) to the concentration in the test water ($C_w$) at steady-state.

(3) "Depuration or clearance or elimination" is the process of losing test material from the test organisms.

(4) "Depuration rate constant ($k_2$)" is the mathematically determined value that is used to define the depuration of test material from previously exposed test animals when placed in untreated dilution water, usually reported in units per hour.

(5) "Steady-state or apparent plateau" is a condition in which the amount of test material being taken up and depurated is equal at a given water concentration.

(6) "Uptake (u)" is the process of sorbing test material into and/or onto the test organisms.

(7) "Uptake phase" is the time during the test when test organisms are being exposed to the test material.

(8) "Uptake rate constant ($k_1$)" is the mathematically determined value that is used to define the uptake of test material by exposed test organisms, usually reported in units of liters/gram/hour.

(c) *Test procedures*—(1) *Summary of the test.* (i) The test compounds' water solubility, n-octanol/water partition coefficient and stability in water (hydrolysis, photolysis and microbial degradation) should be known prior to testing. The 24-hour and 96-hour $LC_{50}$'s for the fish species to be used in the study should also be known. These data should be based on test substance concentrations measured during a flow-through acute toxicity test.

(ii) The procedure proposed is applicable to organic chemicals that are not readily degradable in a microbial degradation test, relatively stable in the aquatic environment and soluble in water at $<1$ mg/l.

(iii) Before any biological experiments are carried out, the analytical method for the particular substance should be tested. It should be shown experimentally on both water and organisms that the recovery as well as the reproducibility are satisfactory. Blank samples (of water, solvents, etc.) should regularly be analyzed to ensure that no contamination occurs. The detection level should be determined and no quantification should be based on signals which are less than 2.5 times the instrument noise. Organisms and water samples should be removed in such a way that no contamination or losses by adsorption occur.

(iv) This guideline describes a procedure for characterizing the bioconcentration potential of chemicals in aquatic biota. Parameters used to characterize the bioconcentration potential include the uptake rate constant ($k_1$), the depuration rate constant ($k_2$), and the steady-state bioconcentration factor, BCF ($k_1/k_2$).

(v) Each of three separate groups of test organisms of the same species is exposed to a different concentration of the test material in water; 0, x, and 10x mg/l, where x is defined by analytical and toxicological boundaries.

The duration of the uptake phase (3 hours to 30 days) and depuration phase (6 hours to 60 days) varies according to the time required to reach the desirable percent of steady-state which is roughly estimated before the test starts. During both phases of the test, organisms and water are periodically removed from the test chambers and analyzed for the test material.

(vi) The uptake rate constant, depuration rate constant(s), bioconcentration factor, and their confidence limits are calculated from the model that best describes the measured concentrations of test material in the organisms and water at any point in time.

(2)—(3) [Reserved]

(4) *Definitive test*—(i) *Test solution preparation.* The test material should be added to the dilution water with minimal use of solvents or other carriers. Several systems adaptable to flow-through tests have been described for saturation of water with relatively insoluble test materials (Chadwick and Kugemgi (1968) under paragraph (f)(3) of this section, Borthwick et al. (1977) under paragraph (f)(2) of this section, Veith and Comstock (1975) under paragraph (f)(11) of this section. Acetone, dimethylformamide, ethanol, methanol, and triethylene glycol are the solvents recommended for use in preparing stock solutions. The concentration of solvent in any test solution should not exceed 0.1 ml/liter in flow-through tests.

(ii) *Exposure concentrations.* Test fish should be exposed to two or more concentrations of test material in water under flow-through conditions. As a guidance, the highest concentration should be less than one-tenth of the threshold or incipient $LC_{50}$ for the test species and at least 10 times higher than the detection limit in water and, if possible, each exposure concentration should differ from another by a factor of 10.

(iii) *Test duration*—(A) *Estimation of the uptake phase.* (*1*) As a guideline, the statistically optimum duration of the uptake phase (u) is near the midpoint of an uptake curve plotted on semi-log paper, or $u=1.6/k_2$, but not more than $3.0/k_2$, which is equivalent to 95 percent of steady-state (Reilly et al. (1977) under paragraph (f)(7) of this section. A pre-test estimate of $k_2$ may be obtained from:

(*i*) A test with the same compound and a different species.

(*ii*) A test with a similar material.

(*iii*) The results of a preliminary range-finding test.

(*iv*) Water solubility data.

$\log k_2=0.43\log(s)-2.11$
where (s) is the aqueous solubility in ppm.

(*v*) *n*-octanol/water partition coefficient data,

$\log k_2=-0.414\log(K_{ow})+0.122$
where $(K_{ow})$ is the n-octanol/water partition coefficient.

(*2*) The duration of the uptake phase (u) for a test material with $\log K_{ow}=3$ would be:

$\log k_2=0.414(3)+0.122=-1.12$
$k_2=0.0759$
$u=1.6/0.0759=21$ hours

Similarly, for a test material having a $\log K_{ow}=6$, the duration of the uptake phase (u) would be:

$\log k_2=-0.414(6)+0.122=-2.362$
$k_2=0.0043$
$u=1.6/0.0043=372$ hours (16 days)

(B) *Estimation of the depuration phase.* Two times u is usually sufficient time for about 95 percent removal of the body burden ($t_{1/2}=0.69/k_2$), but several biological or analytical factors may suggest equally acceptable guidelines. Some compounds follow more complex uptake/depuration behavior than a simple two compartment ($C_w$ and $C_f$), two parameter ($k_1$ and $k_2$) model. For these compounds, longer depuration periods are advisable. On the other hand, the depuration time will most likely be restricted by the lower limit of analytical detection for fish.

(iv) *Sampling schedule.* As a guideline, no fewer than four uptake sampling times and five depuration sampling times should be spaced throughout the duration of the experiment according to the following fractions of the total time ($T_t$): first at 0.0278 $T_t$, second at 0.0556 $T_t$, third at 0.1111 $T_t$, fourth at 0.2222 $T_t$, fifth at 0.3333 $T_t$ (this is the optimum change-over time), sixth at 0.5000 $T_t$, seventh at 0.6667 $T_t$, eighth at 0.8333 $T_t$, and ninth at 1.000 $T_t$. The following Table

I contains examples of acceptable sampling schedules for bioconcentration tests with test materials with a log $K_{ow}=3.0$ and Log $K_{ow}=6.0$:

TABLE I—ACCEPTABLE SAMPLING SCHEDULES FOR BIOCONCENTRATION TESTS

| Test phase | Action | Sampling times log Kow=3 Hours | Sampling times log Kow=6 Days | Number of water samples | Number of fish samples |
|---|---|---|---|---|---|
| | Start test | | | | |
| | | −1 | −2 | | |
| | Add fish | 0 | −1 | 2[1] | |
| | 0.5 | 0 | 2 | | |
| | 1.0 | 0.5 | 2 | | |
| | 1.5 | 1.0 | 2 | | |
| Uptake | 2 | 1.4 | 2 | 4 | |
| | 4 | 2.8 | 2 | 4 | |
| | 7 | 5.7 | 2 | 4 | |
| | 13 | 11 | 2 | 4 | |
| | 20 | 17 | 2 | 6 | |
| | Transfer fish to untreated water 24. | 20 | 2 | | |
| | 30 | 26 | 2 | 4 | |
| Depuration. | 40 | 34 | 2 | 4 | |
| | 50 | 43 | 2 | 4 | |
| Terminate test. | 60 | 51 | 2 | 6 | |
| Total | | | 28 | 40 | |

[1] Samples taken after a minimum of 3 tank volumes have been delivered.

(v) *Sampling procedures.* (A) It is advisable to analyze both water and organism samples as soon as possible after they have been collected to prevent degradation or loss of test material and to determine approximate uptake and depuration rate constants as the test proceeds. If samples cannot be analyzed immediately, it is sometimes appropriate to extract the test material into a solvent, rendering it inert or easier to store until it can be analyzed.

(B) Water samples should be obtained by siphoning through glass tubing from the most central point in the test tank. The sample vessel and siphon should be rinsed with the test solution before collecting the sample.

(C) Water samples are best collected directly into glass vessels of appropriate volume from which the test material can be extracted or analyzed. These vessels might include separatory funnels in the case of organic compounds, or scintillation vials for radioactive test materials.

(D) If significant amounts of particulate matter are present in the water sampled, a second sample should be taken and analyzed after centrifuging to determine whether test material was adsorbed on the particulate matter rather than dissolved.

(E) Water samples containing highly persistent test materials can be stored frozen in plastic containers for later analysis. Care should be exercised to avoid use of containers which could sorb or contaminate samples. With most organic test materials, and especially those tending to degrade easily, a better practice is to extract them from the water and store them under refrigeration in solvent in tightly sealed glass vials.

(F) When removing test organisms for analysis, they should be netted or trapped in a random manner with as little disturbance as possible. If two or more test material concentrations are present, separate nets should be used for each concentration. Organisms should be rinsed with dilution water if accompanied by extraneous matter, blotted dry, and killed by pithing the brain with a dissecting needle or by severing the spinal cord above the opercular region with scissors. They should then be individually weighed and a record made to permit association of the weight with the sample.

(G) Fish may be analyzed as whole fish or as portions, e.g., edible portion (muscle), viscera, remaining carcass, etc. Specific organs may also be analyzed if sufficient biomass is available. If results based on body portions are desired, after the fish is killed it should be eviscerated, taking care not to puncture any parts of the visceral portion which could leak body fluids and possibly cause contamination of the remaining portions. The edible portion or muscle may be removed with a scalpel, blotted dry, and weighed before storing or analysis. The remaining carcass should be weighed before being stored or analyzed. It is necessary to record data for each portion for each individual fish so that whole body data can be reconstructed based on the sum of the parts.

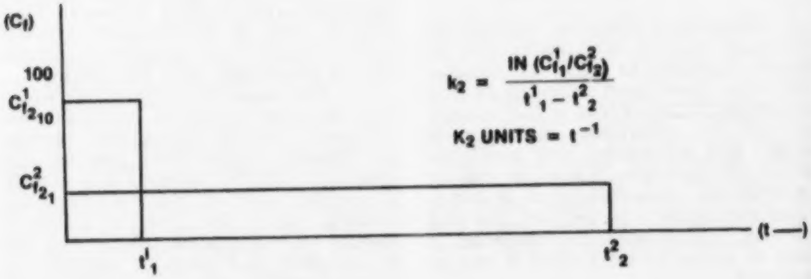
(H) After weighing, the sample is usually ground or homogenized to promote extraction of test material or to enhance solution of the tissue. Procedures for grinding, extraction, separation of impurities, determination of lipid content, etc., are described in the U.S. Food and Drug Administration's Pesticide Analytical Manual (1975) under paragraph (f)(10) of this section or the U.S. Environmental Protection Agency's Manual of Analytical Methods for the Analysis of Pesticide Residues in Human and Environmental Samples (1974) under paragraph (f)(9) of this section.

(I) When determining the bioconcentration of test materials which concentrate in lipids, it is often desirable to determine the percent of the total tissue weight made up by lipids. Results between samples are frequently less variable when based on lipid weight rather than on total weight (Reinert 1970, under paragraph (f)(8) of this section).

(J) Organism samples can be wrapped in acetone-rinsed foil, placed in glass jars and frozen if they are not to be analyzed immediately.

(5) *Test results.* (i) Most bioconcentration data can reasonably be described with a simple two-compartment/two-parameter model as shown by a straight line depuration profile plotted on semi-log paper. If the depuration profile does not appear to be a straight line, then more complex models can be employed (Blau et al. 1975 under paragraph (f)(1) of this section). Typical variations from the simple model include a third parameter to describe the rate of metabolism of the parent compound or two additional parameters to describe redistribution of the parent compound within the body of the fish. If the best model is in question, it may be worthwhile to estimate parameters for the models in question and to compare the likelihood index of each model according to statistical tests (Blau et al. 1975 under paragraph (f)(1) of this section).

(ii) *Graph paper method for depuration rate constant.* Plot each concentration of the test material found in fish at each sampling time on semi-log paper. The slope of that line is $K_2$:



(iii) *Graph paper method for uptake rate constant.* Given $k_2$, calculate $k_1$ as follows:

$$k_1 = \frac{C_{f\,t}}{C_w\,(1-e^{-k_2 t})}$$

The value of $C_f$ is read from the smooth uptake/depuration curve near the uptake mid-point on semi-log paper.

(iv) *Computer method for calculating uptake and depuration rate constant.* The preferred means for obtaining the bioconcentration factor and $k_1$ and $k_2$ rate constants is to use nonlinear parameter estimation methods on a digital computer. Two such programs are BIOFAC (Dow) and NONLIN (Procter and Gamble). These programs find values for $k_1$ and $k_2$ given a set of sequential time concentration data and the model:

$$C_f = \begin{cases} C_w \cdot \dfrac{k_1}{k_2} \left(1-e^{-k_2 t}\right) & 0 \leq t \leq t_c \\ C_w \cdot \dfrac{k_1}{k_2} \left[e^{-k_2(t-t_c)} - e^{-k_2 t_c}\right] & t_c \leq t \end{cases}$$

This approach provides standard deviation estimates of $k_1$ and $k_2$, and BIOFAC statistically weights the analytical and biological variation of the fish concentration data. These and other nonlinear parameter estimation programs are readily available for most computers accepting the Fortran IV language or can be made available from a time-sharing service bureau; they are currently being used by many bioconcentration testing laboratories.

(v) *Validity of the test results.* (A) Scientific judgment rather than rigid criteria should be exercised in accepting or rejecting bioconcentration test results.

(B) Calculated BCF values based on an octanol/water partition coefficient have a very wide confidence margin (greater than ±100 percent), but the quality of the value may be better (narrower confidence margin) than an experimental value from a poorly designed study. Generally, the confidence margins for well designed studies approach ±20 percent. Acceptable bioconcentration data should be reported with confidence margins.

(C) Other criteria for judging the quality of bioconcentration data include the following guidelines:

(*1*) Percent mortality or adverse effect in control or treated organisms (suggested guideline, 10 percent).

(*2*) Percent effect of dose on uptake/depuration rate constants (suggested guideline 20 percent).

(*3*) Percent variation in $C_w$ (suggested guideline, 20 percent) except for the initial dip that may approach 50 percent during the first few days of exposure.

(*4*) Temperature and dissolved oxygen should not vary more than ± 1 °C and ±3 mg/liter.

(*5*) The importance of actually visualizing an apparent plateau has been a subject of recent debate. It is suggested that 80 percent of steady-state ($k_1/k_2$) in any tissue with a confidence margin of ±20 percent is more than sufficient to estimate high quality rate constants for compounds with BCF <10,000. For compounds with BCF >10,000 it may be desirable and acceptable to terminate the uptake phase after a few days not to exceed 28 days even though <80 percent of steady-state was reached.

(*6*) A clearly defined uptake/depuration profile is an indicator of high quality bioconcentration data.

(6) *Analytical measurements.* (i) Prior to analyzing fish or water for the test substance, control samples should be spiked with several different concentrations of the test substance and then analyzed. Final values of $C_w$ and $C_f$ should be corrected for recoveries and background.

(ii) Analytical detection limits of test substance in both fish and water should be determined before the bioconcentration test begins and should be documented in the protocol. As a guideline, the limit of detection may be defined as a signal 2.5 times higher than the background noise level.

(iii) If possible, results reported as "not detected at the limit of detection" should be minimized by pre-test method development and experimental design. These results cannot be used for rate constant calculations. The units $C_w$ and $C_f$ should both be expressed either as ppm or ppb.

(d) *Test conditions—Test species*—(1) *Selection*. (i) The procedures regarding selection of which species to test, their source, handling, holding, disease treatment, acclimation, and quality assurance prior to and during testing should be those given in Committee on Methods for Toxicity Tests with Aquatic Organisms (1975), under paragraph (f)(4) of this section.

(ii) The freshwater fish species used most frequently in bioconcentration tests have been rainbow trout, bluegill, and fathead minnows. The most commonly used marine fish have been spot, sheepshead minnows, silversides, shiner perch, English sole, staghorn sculpin, and 3-spine sticklebacks. These species are more readily available than most others and can be obtained in convenient sizes.

(2) *Facilities*—(i) *Construction materials*. Construction materials and commercially purchased equipment that may contact any water into which test organisms are placed should not contain any substances that can be leached or dissolved by the water. Glass, #316 stainless steel, and perfluorocarbon plastics should be used whenever possible to minimize leaching, dissolution, and sorption. Some will be more suitable than others for use with specific test materials. Unplasticized plastics, cast iron, and concrete can be used for holding and acclimation tanks and in the water supply system. Rubber, copper, brass, galvanized metal, and lead should not come into contact with dilution water, stock solutions, effluent samples or test solutions.

(ii) *Toxicant delivery system*. (A) One of several toxicant delivery systems can be used successfully, including the proportional diluter (Lemke et al. 1977), under paragraph (f)(6) of this section. Diluters are accurate over extended periods of time, are relatively trouble-free, and have fail-safe provisions. However, proportional diluters often require that laboratories have more than 8 feet of headroom. A small chamber to promote mixing of test material-bearing and dilution water should be used between the diluter and test chambers for each concentration. Design alterations, such as modification to deliver duplicates of only two or three concentrations, are easy to make (Jarvinen et al. 1977, under paragraph (f)(5) of this section).

(B) Pump systems are relatively simple to understand and use, require little space, and can be quite accurate. Some investigators have found metering pumps to maintain less variable test concentrations than piston operated pumps.

(C) The performance of the toxicant delivery system should be checked before and during each test. This should include determination of the flow rate through each test chamber and measurement of either the concentration of toxicant in each test chamber or the volumes delivered by each portion of the delivery system. The general operation of the toxicant delivery system should be checked daily during the test.

(D) The flow rate through the test chambers should be at least five volume additions per 24 hours, but should take into account the size of the test chamber, the size of the test organisms and the loading. It is usually desirable to construct the metering system so that it can provide at least 10 volume additions per 24 hours. The flow rates through the test chambers should not vary by more than 20 percent from any one test chamber to any other or from one time to another within a test.

(iii) *Test chambers*. Each of the height and width dimensions of the test chamber should be at least 1.5 times the largest horizontal dimension of the test organism. A minimum volume of one liter per fish is crowded but satisfactory for fish up to 15 g; large volumes to fish weight ratios are preferred to minimize the initial $C_w$ dip and to help maintain the dissolved oxygen concentration.

(iv) *Cleaning.* Metering systems, test chambers, and equipment used to prepare and store dilution water, stock solutions, and test solutions should be cleaned before use. New equipment should be washed with detergent and rinsed with water, pesticide-free acetone, water, acid (such as 5 percent concentrated nitric acid), and twice with distilled water. At the end of every test, all items that are to be used again should be immediately emptied, rinsed with water, cleaned by a procedure appropriate for removing the test material (e.g., acid to remove metals and bases; detergent, organic solvent, or activated carbon to remove organic compounds), and rinsed twice with distilled water. Acid is useful for removing mineral deposits, and 200 mg of hypochlorite/liter is useful for removing organic matter and for disinfection. A solution containing 200 mg hypochlorite per liter is conveniently prepared by adding 6 ml of liquid household chlorine bleach to 1 liter of water. However, acid and hypochlorite should not be mixed because hazardous fumes may be produced. Metering systems and test chambers should be rinsed with dilution water just before use.

(v) *Dilution water*—(A) *General requirements.* (*1*) An adequate supply of dilution water that is acceptable to the test organisms and to the purpose of the test should be available. A minimum criterion for an acceptable dilution water is that healthy test organisms will survive in it for the duration of acclimation and testing without showing signs of stress, such as discoloration or unusual behavior. A better criterion for an acceptable freshwater dillution water is that test organisms will survive, grow, and reproduce satisfactorily in it.

(*2*) If the dilution water is or is prepared from dechlorinated water, it should be shown that in fresh samples of the dilution water either (*i*) the concentration of residual chlorine is less than 3 mg/liter or (*ii*) *Acartia tonsa*, mysid shrimp, oyster larvae, or first instar daphnids can survive for 48 hours without food. The dilution water should be assayed for the selected test material.

(B) *Freshwater.* Because daphnids are more sensitive to many toxicants than most other readily available freshwater aquatic animals, water in which first instar daphnids will survive for 48 hours without food is probably acceptable for most short-term tests with freshwater animals. Water in which daphnids will survive, grow, and reproduce satisfactorily should be an acceptable dilution water for longer tests with freshwater animals.

(C) *Estuarine and marine water.* Because *Acartia tonsa*, mysid shrimp, and oyster larvae are more sensitive to many toxicants than most other estuarine and marine aquatic animals, water in which they will survive for 48 hours without food is probably acceptable for most short-term tests with estuarine and marine animals. Water in which *Acartia tonsa* or mysid shrimp will survive, grow, and reproduce satisfactorily should be an acceptable dilution water for longer tests with estuarine and marine animals.

(e) *Reporting.* In addition to the reporting requirements prescribed in Part 792—*Good Laboratory Practice Standards* of this chapter, the test report should include the following information:

(1) A detailed description of the test material, including its source, lot number, composition (identity and concentration of major ingredients and major impurities), known physical and chemical properties, and identity and concentration of any carriers (solvents) or other additives used.

(2) The source of the dilution water, its chemical characteristics, and a description of any pretreatment.

(3) Detailed information about the test organisms, including scientific name and how verified (and strain for salmonids when appropriate), weight (wet, blotted dry), standard length of fish, height of bivalve molluscs, age, life stage, source, history, observed diseases, treatments, acclimation procedure, and food used.

(4) A description of the experimental design and metering system.

(5) Description of tissue and water samples analyzed, and methods used to obtain, prepare, and store them.

(6) Methods used for, and results (with standard deviation) of all chemi-

cal analyses of water quality and concentration of test material in tissue and water, including validation studies and reagent blanks.

(7) The steady-state bioconcentration factor, the uptake and depuration rate constants, the confidence margins (± standard deviation) and the method of computations/data analysis.

(8) Anything unusual about the test, any deviation from these procedures, and any other relevant information.

(f) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Blau, G.E., Neely, W.B., Branson, D.R. "Ecokinetics: a study of the fate and distribution of chemicals in laboratory ecosystems," *American Institute of Chemical Engineering Journal,* 21:854-861 (1975).

(2) Borthwick, P.W., Tagatz, M.E., Forester, J. "A gravity-flow column to provide pesticide-laden water for aquatic bioassays," *Bulletin of Environmental Contamination and Toxicology,* 13:183-187 (1977).

(3) Chadwick, G.C., Kugemagi, V. "Toxicity evaluation of a technique for introducing dieldrin into water," *Journal of the Water Pollution Control Federation,* 40: 76-82 (1968).

(4) U.S. Environmental Protection Agency, Committee on Methods for Toxicity Tests with Aquatic Organisms. "Methods for acute toxicity tests with fish, macroinvertebrates, and amphibians," EPA Report 660/3-75-009 (Corvallis, Oregon, 1975).

(5) U.S. Environmental Protection Agency, Jarvinen, A.W., Hoffman, M.J., Thorslund, T.W. "Toxicity of DDT food and water exposure to fathead minnows" U.S. Environmental Protection Agency. EPA Report No. 600/3-76-114 (Duluth, Minnesota, 1977).

(6) U.S. Environmental Protection Agency, Lemke, A.E., Brungs, W.A., Halligan, B.J. "Manual for construction and operation of toxicity testing proportional diluters," EPA Report 600/3-78-072 (Duluth, Minnesota, 1978).

(7) Reilly, P.M., Bajramovic, R., Blau, G.E., Branson, D.R., Sauerhoff, M.W. "Guidelines for the optimal design of experiments to estimate parameters in first order kinetic models," *Canadian Journal of Chemical Engineering,* 55:614-622 (1977).

(8) Reinert, R.E. "Pesticide concentrations in Great Lakes fish," *Pesticides Monitoring Journal,* 3(4): 233-240 (1970).

(9) U.S. Environmental Protection Agency. "Analysis of human or animal adipose tissue," Ed. Thompson, J.F., *Analyses of Pesticide Residues in Human and Environmental Samples,* 1974, available from U.S. Environmental Protection Agency, Research Triangle Park, NC 27711.

(10) U.S. Food and Drug Administration. *Pesticide Analytical Manual.* Vol. 1, 1975, available from the Food and Drug Administration, 5600 Fishers Lane, Rockville, MD 20852.

(11) Veith, G.D., Comstock, V.M. "Apparatus for continuously saturating water with hydrophobic organic chemicals" *Journal of Fishery Research Board of Canada,* 32: 1849-1851 (1975).

[50 FR 39321, Sept. 27, 1985]

## § 797.1600 Fish early life stage toxicity test.

(a) *Purpose.* This guideline is intended to be used for assessing the propensity of chemical substances to produce adverse effects to fish during the early stages of their growth and development. This guideline describes the conditions and procedures for the continuous exposure of several representative species to a chemical substance during egg, fry and early juvenile life stages. The Environmental Protection Agency (EPA) will use data from this test in assessing the potential hazard of the test substance to the aquatic environment.

(b) *Definitions.* The definitions in section 3 of the Toxic Substances Control Act (TSCA) and the definitions in Part 792—*Good Laboratory Practice Standards,* apply to this section. In addition, the following definitions are applicable to this specific test guideline:

(1) "Acclimation" physiological or behavioral adaptation of organisms to one or more environmental conditions

associated with the test method (e.g., temperature, hardness, pH).

(2) "Carrier" solvent or other agent used to dissolve or improve the solubility of the test substance in dilution water.

(3) "Conditioning" exposure of construction materials, test chambers, and testing apparatus to dilution water or to the test solution prior to the start of the test in order to minimize the sorption of test substance onto the test facilities or the leachig of substances from test facilities into the dilution water or the test solution.

(4) "Control" an exposure of test organisms to dilution water only or dilution water containing the test solvent or carrier (no toxic agent is intentionally or inadvertently added).

(5) "Dilution water" the water used to produce the flow-through conditions of the test to which the test substance is added and to which the test species is exposed.

(6) "Early life stage toxicity test" a test to determine the minimum concentration of a substance which produces a statistically significant observable effect on hatching, survival, development and/or growth of a fish species continuously exposed during the period of their early development.

(7) "Embryo cup" a small glass jar or similar container with a screened bottom in which the embryos of some species (i.e., minnow) are placed during the incubation period and which is normally oscillated to ensure a flow of water through the cup.

(8) "Flow through" refers to the continuous or very frequent passage of fresh test solution through a test chamber with no recycling.

(9) "Hardness" the total concentration of the calcium and magnesium ions in water expressed as calcium carbonate (mg $CaCO_3$/liter).

(10) "Loading" the ratio of biomass (grams of fish, wet weight) to the volume (liters) of test solution passing through the test chamber during a specific interval (normally a 24-hr. period).

(11) "No observed effect concentration (NOEC)" the highest tested concentration in an acceptable early life stage test: (i) which did not cause the occurrence of any specified adverse effect (statistically different from the control at the 95 percent level); and (ii) below which no tested concentration caused such an occurrence.

(12) "Observed effect concentration (OEC)" the lowest tested concentration in an acceptable early life stage test: (i) Which caused the occurrence of any specified adverse effect (statistically different from the control at the 95 percent level); and (ii) above which all tested concentrations caused such an occurrence.

(13) "Replicate" two or more duplicate tests, samples, organisms, concentrations, or exposure chambers.

(14) "Stock solution" the source of the test solution prepared by dissolving the test substance in dilution water or a carrier which is then added to dilution water at a specified, selected concentration by means of the test substance delivery system.

(15) "Test chamber" the individual containers in which test organisms are maintained during exposure to test solution.

(16) "Test solution" dilution water with a test substance dissolved or suspended in it.

(17) "Test substance" the specific form of a chemical substance or mixture that is used to develop data.

(c) *Test Procedures*—(1) *Summary of test.* (i) The early life stage toxicity test with fish involves exposure of newly fertilized embryos to various concentrations of a test substance. Exposure continues for 28 days post hatch for the minnows and 60 days post hatch for the trout species. During this time various observations and measurements are made in a specific manner and schedule in order to determine the lowest effect and highest no-effect concentrations of the test substance.

(ii) A minimum of five exposure (treatment) concentrations of a test substance and one control are required to conduct an early life stage toxicity test. The concentration of the test substance in each treatment is usually 50 percent of that in the next higher treatment level.

(iii) For each exposure concentration of the test substance and for each control (i.e., regular control and carrier control is required) there shall be:

(A) At least two replicate test chambers, each containing one or more embryo incubation trays or cups; and there shall be no water connections between the replicate test chambers;

(B) At least 60 embryos divided equally in such a manner that test results show no significant bias from the distributions, between the embryo incubation trays or cups for each test concentration and control (i.e., 30 per embryo cup with 2 replicates);

(C) All surviving larvae divided equally between the test chambers for each test concentration and control (e.g., 30 larvae per test chamber with 2 replicates).

(iv) *Duration.* (A) For fathead minnow and sheepshead minnow a test begins when the newly fertilized minnow embryos (less than 48-hours old) are placed in the embryo cups and are exposed to the test solution concentrations. The test terminates following 28 days of post-hatch exposure, i.e., 28 days after the newly hatched fry are transferred from the embryo cups into the test chambers.

(B) For brook trout and rainbow trout a test begins when newly fertilized trout embryos (less than 96-hours old) are placed in the embryo trays or cups and are exposed to the test solution concentrations. The test terminates following 60 days of post-hatch exposure (for an approximate total exposure period of 90 days).

(C) For silverside a test begins with newly fertilized embryos (less than or equal to 48 hours old) and is terminated 28 days after hatching. The chorionic fibrils should be cut before randomly placing the embryos in the egg incubation cups.

(2) [Reserved]

(3) *Range-finding test.* (i) A range finding test is normally performed with the test substance to determine the test concentrations to be used in the early life stage toxicity test, especially when the toxicity is unknown. It is recommended that the test substance concentrations be selected based on information gained from a 4- to 10-day flow-through toxicity test with juveniles of the selected test species.

(ii) The highest concentration selected for the early life stage toxicity test should approximate the lowest concentration indicated in any previous testing to cause a significant reduction in survival. The range of concentrations selected is expected to include both observed effect and no-observed effect levels. The dilution factor between concentrations is normally 0.50, however, other dilution factors may be used as necessary.

(4) *Definitive test*—(i) *General.* (A) A test shall not be initiated until after the test conditions have been met and the test substance delivery system has been observed functioning properly for 48-hours. This includes temperature stability, flow requirements of dilution water, lighting requirements, and the function of strainers and air traps included in the water-supply system, and other conditions as specified previously.

(B) New holding and test facilities should be tested with sensitive organisms (i.e., juvenile test species or daphnids) before use to assure that the facilities or substances possibly leaching from the equipment will not adversely affect the test organisms during an actual test.

(C) Embryos should be acclimated for as long as practical to the test temperature and dilution water prior to the initiation of the test.

(D) When embryos are received from an outside culture source (i.e., rainbow and brook trout) at a temperature at variance with the recommended test temperature they shall be acclimated to the test temperature. When eggs are received, they should be immediately unpacked and the temperature of the surrounding water determined. Sudden temperature changes should be avoided. Acclimation to the appropriate test temperature should be accomplished within a period of 6 hours, and should incorporate the use of dilution water.

(E) Embryos should be visually inspected prior to placement in the embryo cups or screen trays. All dead embryos shall be discarded. Dead embryos can be discerned by a change in coloration from that of living embryos (e.g., trout embryos turn white when dead). During visual inspection, empty shells, opaque embryos, and embryos with fungus or partial shells attached shall be removed and discarded. If less than 50 percent of the eggs to be used appear to be healthy, all embryos in such a lot shall be discarded.

(ii) *Embryo incubation procedures.* (A) Embryos can be distributed to the embryo cups or screen trays using a pipette with a large bore or a similar apparatus. Newly-hatched silverside fry are very sensitive to handling; the egg incubation cups should not be handled at all the first 5 days after hatching begins. Just before hatching is expected to begin, the embryos should be transferred to clean incubation cups. Trout embryos can be distributed by using a small container which has been precalibrated to determine the approximate number of embryos it can hold; embryos are measured volumetrically in this manner, and are then poured onto the screen tray (or embryo cup). Trout embryos should be separated on the screen tray so that they are not in contact with each other. A final count will ensure the actual number on the screen tray. After random assignment, the screen trays or embryo cups are placed in the test chambers.

(B) Each day until hatch the embryos are visually examined. Minnow embryos may be examined with the aid of a magnifying viewer. Trout embryos should not be touched. Trout embryos should be maintained in low intensity light or in darkness until 1-week post hatch, and are usually examined with the aid of a flashlight or under low intensity light. Dead embryos should be removed and discarded. Any embryos which are heavily infected with fungus shall be discarded and shall be subtracted from the initial number of embryos used as a basis for the calculations of percentage hatch.

(C) When embryos begin to hatch they should not be handled.

(iii) *Initiation of fry exposure.* (A) Forty-eight hours after the first hatch in each treatment level, or when hatching is completed, the live young fish shall be counted and transferred from each embryo cup into the appropriate test chamber. For silverside, all surviving fry are not counted until six days after hatching and are not transferred to embryo cups. All of the normal and abnormal fry shall be gently released into the test chamber by allowing the fry to swim out of each embryo cup; nets shall not be used. The trout embryos incubated on screen trays will hatch out in the test chambers, therefore handling of fish is not necessary.

(B) If necessary, fry can be transferred from one replicate embryo cup to the other replicate within a test concentration to achieve equal numbers in each replicate chamber.

(C) The number of live fry, live normal fry, live embryos, dead embryos and unaccounted for embryos for each cup shall be recorded when hatching is deemed complete. Those fry which are visibly (without the use of a dissecting scope or magnifying viewer) lethargic or grossly abnormal (either in swimming behavior or physical appearance) shall be counted. Late hatching embryos shall be left in the embryo cups to determine if they will eventually hatch or not. The range of time-to-hatch (to the nearest day) for each cup shall be recorded.

(iv) *Time to first feeding.* (A) The first feeding for the fathead and sheepshead minnow fry shall begin shortly after transfer of the fry from the embryo cups to the test chambers. Silversides are fed the first day after hatch. Trout species initiate feeding at swim-up. The trout fry shall be fed trout starter mash three times a day *ad libitum*, with excess food siphoned off daily. The minnow fry shall be fed live newly-hatched brine shrimp nauplii (*Artemia salina*) at least three times a day.

(B) For the first seven days, feeding shall be done at minimum intervals of four hours (i.e., 8 am, 12 noon, and 4 pm); thereafter the fry shall be fed as indicated below.

(v) *Feeding.* (A) The fathead and sheepshead minnow fry shall be fed newly hatched brine shrimp nauplii for the duration of the test at approximately 4-hour intervals three times a day during the week and twice on the weekend after the first week. Trout fry shall be fed at similar intervals and may receive live brine shrimp nauplii in addition to the trout starter food after the first week. Between days 1 and 8 after first hatching, silverside fry are fed the rotifer, *Brachionus plicatilis*, three times daily at a concentration of 5,000 to 10,000 organisms per egg cup (based on 15 fish/cup). From days 9 to 11, the fry shall be fed approximately 2,500 newly hatched brine shrimp (*Artemia*) nauplii and 5,000 to 10,000 rotifers twice daily. For the remainder of the test, the fish will be fed brine shrimp exclusively. The number of organisms used should be gradually increased to approximately 5,000 nauplii by test day 28.

(B) An identical amount of food should be provided to each chamber. Fish should be fed *ad libitum* for 30 minutes with excess food siphoned off the bottom once daily if necessary.

(C) Fish should not be fed for the last 24 hours prior to termination of the test.

(vi) *Carriers.* Water should be used in making up the test stock solutions. If carriers other than water are absolutely necessary, the amount used should be the minimum necessary to achieve solution of the test substance. Triethylene glycol and dimethyl formamide are preferred, but ethanol and acetone can be used if necessary. Carrier concentrations selected should be kept constant at all treatment levels.

(vii) *Controls.* Every test requires a control that consists of the same dilution water, conditions, procedures, and test organisms from the same group used in the other test chambers, except that none of the test substance is added. If a carrier (solvent) is used, a separate carrier control is required in addition to the regular control. The carrier control shall be identical to the regular control except that the highest amount of carrier present in any treatment is added to this control. If the test substance is a mixture, formulation, or commercial product, none of the ingredients is considered a carrier unless an extra amount is used to prepare the stock solution.

(viii) *Randomization.* The location of all test chambers within the test system shall be randomized. A representative sample of the test embryos should be impartially distributed by adding to each cup or screen tray no more than 20 percent of the number of embryos to be placed in each cup or screen tray and repeating the process until each cup or screen tray contains the specified number of embryos. Alternatively, the embryos can be assigned by random assignment of a small group (e.g., 1 to 5) of embryos to each embryo cup or screen tray, followed by random assignment of a second group of equal number to each cup or tray, which is continued until the appropriate number of embryos are contained in each embryo cup or screen tray. The method of randomization used shall be reported.

(ix) *Observations.* During the embryo exposure period observations shall be made to check for mortality. During the exposure period of the fry, observations shall be made to check for mortality and to note the physical appearance and behavior of the young fish. The biological responses are used in combination with physical and chemical data in evaluating the overall lethal and sublethal effects of the test substance. Additional information on the specific methodology for the data obtained during the test procedure are discussed in the following sections.

(x) *Biological data.* (A) Death of embryos shall be recorded daily.

(B) When hatching commences, daily records of the number of embryos remaining in each embryo cup are required. This information is necessary to quantify the hatching success. A record of all deformed larvae shall be kept throughout the entire post-hatch exposure. Time to swim-up shall be recorded for the trout. Upon transfer of fry from the embryo cups to the test chambers, daily counts of the number of live fish should be made. At a minimum, live fish shall be counted on days 4, 11, 18, 25 and (weekly thereafter for the trout species) finally on termination of the test.

(C) The criteria for death of young fish is usually immobility, especially absence of respiratory movement, and lack of reaction to gentle prodding. Deaths should be recorded daily and dead fish removed when discovered.

(D) Daily and at termination of the test, the number of fish that appear (without the use of a magnifying viewer) to be abnormal in behavior (e.g., swimming erratic or uncoordinated, obviously lethargic, hyperventilating, or over excited, etc.) or in physical appearance (e.g., hemorrhaging, producing excessive mucous, or are discolored, deformed, etc.) shall be recorded and reported in detail.

(E) All physical abnormalities (e.g., stunted bodies, scoliosis, etc.) shall be photographed and the deformed fish which die, or are sacrificed at the termination of the test, shall be preserved for possible future pathological examination.

(F) At termination, all surviving fish shall be measured for growth. Standard length measurements should be made directly with a caliper, but may be measured photographically. Measurements shall be made to the nearest millimeter (0.1 mm is desirable). Weight measurements shall also be made for each fish alive at termination (wet, blotted dry, and to the nearest 0.01 g for the minnows and 0.1 g for the trout). If the fish exposed to the toxicant appear to be edematous compared to control fish, determination of dry, rather than wet, weight is recommended.

(G) Special physiological, biochemical and histological investigations on embryos, fry, and juveniles may be deemed appropriate and shall be performed on a case by case basis.

(5) *Test results.* (i) Data from toxicity tests are usually either continuous (e.g. length or weight measurements) or dichotomous (e.g. number hatching or surviving) in nature. Several methods are available and acceptable for statistical analysis of data derived from early life stage toxicity tests; however, the actual statistical methodology to analyze and interpret the test results shall be reported in detail.

(ii) The significance level for all statistical testing shall be a minimum of P=0.05 (95 percent confidence level).

(A) *Example of statistical analysis.* (*1*) Mortality data for the embryonic stage, fry stage and for both stages in replicate exposure chambers should first be analyzed using a two-way analysis of variance (ANOVA) with interaction model. This analysis will determine if replicates are significantly different from each other. If a significant difference between replicates or a significant interaction exists, cause for the difference should be determined. Modification should then be made in the test apparatus or in handling procedures for future toxicity tests. Further calculations should incorporate the separation of replicates. If no significant difference is observed, replicates may be pooled in further analyses.

(*2*) After consideration of replicate responses, mortality data should then be subjected to one-way ANOVA. The purpose of this analysis is to determine if a significant difference exists in the percentage mortality between control fish and those exposed to the test material.

(*3*) If the one-way ANOVA results in a F ratio that is significant, it would be acceptable to perform t-tests on the control versus each concentration. A second technique is to identify treatment means that are significantly different; this method should involve the additional assumption that the true mean response decreases generally with increasing concentration. The researcher may also be interested in determining significant differences between concentrations.

(*4*) Growth data should also be analyzed by one-way ANOVA with the inclusion of a covariate to account for possible differences in growth of surviving fry in embryo cup(s) that contain fewer individuals. This condition can occur in cases when the same amount of food is given to each test chamber regardless of the number of survivors.

(B) *Test data to be analyzed.* Data to be statistically analyzed are:

(*1*) Percentage of healthy, fertile embryos at 40–48 hours after initiation of the test. Percentage is based upon initial number used.

(*2*) Percentage of embryos that produce live fry for release into test chambers. Percentage is based on number of embryos remaining after thinning.

(*3*) Percentage of embryos that produce live, normal fry for release into test chambers. Percentage is based upon number of embryos remaining after thinning.

(*4*) Percentage of fry survival at swim-up for trout. Percentage is based upon number of embryos remaining after thinning.

(*5*) Percentage of embryos that produce live fish at end of test. Percentage is based upon number of embryos remaining after thinning.

(*6*) Percentage of embryos that produce live, normal fish at end of test. Percentage is based upon number of embryos remaining after thinning.

(*7*) Weights and lengths of individual fish alive at the end of the test.

(C) It is important that fish length and weight measurements be associated with individual test chambers since the density of the fish and available food should be considered in the growth of the organism.

(iii) *Acceptability criteria.* (A) An early life stage toxicity test is not acceptable unless at least one of the following criteria is significantly different ($p=0.05$) from control organisms when compared with treated organisms, and the responses are concentration-dependent: mortality of embryos, hatching success, mortality of fry (at swim-up for trout), total mortality throughout the test, and growth (i.e. weight). If no significant effects occur, but the concentrations tested were the highest possible due to solubility or other physio-chemical limitations, the data will be considered for acceptance.

(B) In addition to obtaining significant effects on the exposed test species, a measure of acceptability in the response of control fish is also required.

(C) A test is not acceptable if the average survival of the control fish at the end of the test is less than 80 percent or if survival in any one control chamber is less than 70 percent. For silversides, a test is not acceptable if the average overall survival of the control embryos and fish at the end of the test is less than 60 percent.

(D) If a carrier is used, the criteria for effect (mortality of embryos and fry, growth, etc.) used in the comparison of control and exposed test organisms shall also be applied to the control and control with carrier chambers. For the test to be considered acceptable, no significant difference shall exist between these criteria.

(E) A test is not acceptable if the relative standard deviation (RSD=100 times the standard deviation divided by the mean) of the weights of the fish that were alive at the end of the test in any control test chamber is greater than 40 percent.

(6) *Analytical measurements*—(i) *Analysis of water quality.* Measurement of certain dilution water quality parameters shall be performed every 6 months, to determine the consistency of the dilution water quality. In addition, if data in 30-day increments are not available to show that freshwater dilution water is constant, measurements of hardness, alkalinity, pH, acidity, conductivity, TOC or COD and particulate matter should be conducted once a week in the highest test substance concentration. Measurement of calcium, magnesium, sodium, potassium, chloride, and sulfate is desirable.

(ii) *Dissolved oxygen measurement.* The dissolved oxygen concentration shall be measured in each test chamber at the beginning of the test and at least once weekly thereafter (as long as live organisms are present) in two replicates of the control and the high, medium, and low test substance concentrations.

(iii) *Temperature measurement.* Temperatures shall be recorded in all test chambers at the beginning of the test, once weekly thereafter and at least hourly in one test chamber. When possible, the hourly measurement shall be alternated between test chambers and between replicates.

(iv) *Test substance measurement.* (A) Prior to the addition of the test substance to the dilution water, it is recommended that the test substance stock solution be analyzed to verify the concentration. After addition of the test substance, the concentration of test substance should be measured at the beginning of the test in each test concentration and control(s), and at least once a week thereafter. Equal aliquots of test solution may be removed from each replicate chamber and pooled for analysis. If a malfunction in the delivery system is discovered, water samples shall be taken from the affected test chambers immediately and analyzed.

(B) The measured concentration of test substance in any chamber should be no more than 30 percent higher or lower than the concentration calculated from the composition of the stock solution and the calibration of the test substance delivery system. If the difference is more than 30 percent, the concentration of test substance in the solution flowing into the exposure chamber (influent) should be analyzed. These results will indicate whether the problem is in the stock solution, the test substance delivery system or in the test chamber. Measurement of degradation products of the test substance is recommended if a reduction of the test substance concentration occurs in the test chamber.

(v) *Sampling and analysis methodology.* (A) Generally, total test substance measurements are sufficient; however, the chemical characteristics of the test substance may require both dissolved and suspended test substance measurements.

(B) For measurement of the test substance, water samples shall be taken midway between the top, bottom, and sides of the test chamber and should not include any surface scum or material stirred up from the bottom or sides. Samples of test solutions shall be handled and stored appropriately to minimize loss of test substance by microbial degradation, photodegradation, chemical reaction, volatilization, or sorption.

(C) Chemical and physical analyses shall be performed using standardized methods whenever possible. The analytical method used to measure the concentration of the test substance in the test solution shall be validated before the beginning of the test. At a minimum, a measure of the accuracy of the method should be obtained on each of two separate days by using the method of known additions, and using dilution water from a tank containing test organisms. Three samples should be analyzed at the next-to-lowest test substance concentration. It is also desirable to study the accuracy and precision of the analytical method for test guideline determination by use of reference (split) samples, or interlaboratory studies, and by comparison with alternative, reference, or corroborative methods of analysis.

(D) An analytical method is not acceptable if likely degradation products of the test substance, such as hydrolysis and oxidation products, give positive or negative interferences, unless it is shown that such degradation products are not present in the test chambers during the test. In general, atomic absorption spectrophotometric methods for metals and gas chromatographic methods for organic compounds are preferable to colorimetric methods.

(E) In addition to analyzing samples of test solution, at least one reagent blank also should be analyzed when a reagent is used in the analysis. Also, at least one sample for the method of known additions should be prepared by adding test substance at the concentration used in the toxicity test.

(d) *Test conditions*—(1) *Test species.* (i) One or more of the recommended test species will be specified in rules under Part 799 of this chapter requiring testing of specific chemicals. The recommended test species are:

(A) Fathead minnow (*Pimephales promelas* Rafinesque).

(B) Sheepshead minnow (*Cyprinodon variegatus*).

(C) Brook trout ( *Salvelinus fontinalis*).

(D) Rainbow trout (*Salmo gairdneri*).

(E) Atlantic silverside (*Menidia menidia*).

(F) Tidewater silverside (*Menidia peninsulae*).

(ii) Embryos used to initiate the early life stage test shall be less than 48 hours old for the fathead and sheepshead minnows, silversides, and less than 96 hours old for the brook trout and rainbow trout. In addition, the following requirements shall be met:

(A) All embryos used in the test shall be from the same source. Embryos shall be obtained from a stock cultured in-house when possible, and maintained under the same parameters as specified for the test conditions. When it is necessary to obtain embryos from an external source, caution should be exercised to ensure embryo viability and to minimize the possibility of fungal growth. A description of the brood stock history or embryo source shall be made available to EPA upon request.

(B) Test species shall be cared for and handled properly in order to avoid unnecessary stress. To maintain test species in good condition and to maximize growth, crowding shall be prevented, and the dissolved oxygen level shall be maintained near saturation.

(C) Embryos and fish shall be handled as little as possible. Embryos shall be counted and periodically inspected until hatching begins. When larvae begin to hatch, they shall not be handled. Transfer of minnow larvae from embryo cups to test chambers shall not involve the use of nets. No handling is necessary following introduction into the test chambers until termination of the test.

(D) If fathead minnow embryos are obtained from in-house culture units, the embryos should be gently removed from the spawning substrate. The method for separating the fertilized eggs from the substrate is important and can affect the viability of the embryos; therefore the finger-rolling procedure is recommended.

(E) Disease treatment. Chemical treatments to cure or prevent diseases should not be used before, and should not be used during a test. All prior treatments of brood stock should be reported in detail. Severely diseased organisms should be destroyed.

(2) *Test facilities*—(i) *Construction materials*. Construction materials and equipment that contact stock solutions, test solutions, or dilution water into which test embryos or fish are placed should not contain any substances that can be leached or dissolved into aqueous solutions in quantities that can affect test results. Materials and equipment that contact stock or test solutions should be chosen to minimize sorption of test chemicals from dilution water. Glass, #316 stainless steel, nylon screen and perfluorocarbon plastic (e.g., Teflon®) are acceptable materials. Concrete or rigid (unplasticized) plastic may be used for holding and acclimation tanks, and for water supply systems, but they should be thoroughly conditioned before use. If cast iron pipe is used in freshwater supply systems, colloidal iron may leach into the dilution water and strainers should be used to remove rust particles. Natural rubber, copper, brass, galvanized metal, epoxy glues, and flexible tubing should not come in contact with dilution water, stock solutions, or test solutions.

(ii) *Test chambers* (exposure chambers). (A) Stainless steel test chambers should be welded or glued with silicone adhesive, and not soldered. Glass should be fused or bonded using clear silicone adhesive. Epoxy glues are not recommended, but if used ample curing time should be allowed prior to use. As little adhesive as possible should be in contact with the water.

(B) Many different sizes of test chambers have been used successfully. The size, shape and depth of the test chamber is acceptable if the specified flow rate and loading requirements can be achieved.

(C) The actual arrangement of the test chambers can be important to the statistical analysis of the test data. Test chambers can be arranged totally on one level (tier) side by side, or on two levels with each level having one of the replicate test substance concentrations or controls. Regardless of the arrangement, it shall be reported in detail and considered in the data analysis.

(iii) *Embryo incubation apparatus*. (A) Recommended embryo incubation apparatus include embryo cups for the minnow species and screen trays for the trout species, although embryo cups can be used for the trout species. Embryo cups are normally constructed from approximately 4–5 cm inside diameter, 7–8 cm high, glass jars with the end cut off or similar sized sections of polyethylene tubing. One end of the jar or tubing is covered with stainless steel or nylon screen (approximately 40 meshes per inch is recommended). Embryo cups for silversides are normally constructed by using silicone adhesive to glue a 10-cm high, 363-um nylon mesh tube inside a 9-cm I.D. glass Petri dish bottom. The embryo cups shall be appropriately labeled and then suspended in the test chamber in such a manner as to ensure that the test solution regularly flows through the cup and that the embryos are always submerged but are not agitated too vigorously. Cups may be oscillated by a rocker arm apparatus with a low rpm motor (e.g., 2 rpm) to maintain the required flow of test water. The vertical-travel distance of the rocker arm apparatus during oscillation is normally 2.5–4.0 cm. The water level in the test chambers may also be varied by means of a self-starting siphon in order to ensure exchange of water in the embryo cups.

(B) The trout embryo incubation trays can be made from stainless steel screen (or other acceptable material such as plastic) of about 3–4 mm mesh. The screen tray should be supported above the bottom of the test chamber by two folds of screen or other devices which function as legs or supports. The edges of the screen tray should be turned up to prevent bump spills and to prevent the embryos from rolling off in the event of excessive turbulence. Suspending or supporting the screen tray off the bottom ensures adequate water circulation around the embryos and avoids contact of embryos with possible bottom debris.

(iv) *Test substance delivery system.* (A) The choice of a specific delivery system depends upon the specific properties and requirements of the test substance. The apparatus used should accurately and precisely deliver the appropriate amount of stock solution and dilution water to the test chambers. The system selected shall be calibrated before each test. Calibration includes determining the flow rate through each chamber, and the proportion of stock solution to dilution water delivered to each chamber. The general operation of the test substance delivery system shall be checked at least twice daily for normal operation throughout the test. A minimum of five test substance concentrations and one control shall be used for each test.

(B) The proportional diluter and modified proportional diluter systems and metering pump systems have proven suitable and have received extensive use.

(C) Mixing chambers shall be used between the diluter and the test chamber(s). This may be a small container or flow-splitting chamber to promote mixing of test substance stock solution and dilution water, and is positioned between the diluter and the test chambers for each concentration. If a proportional diluter is used, separate delivery tubes shall run from the flow-splitting chamber to each replicate test chamber. Daily checks on this latter system shall be made.

(D) Silverside fry are injured easily and are susceptible to impingement on the mesh of the incubation cups. Consequently, water flow into and out of the cups when counting fry must be at a slow rate. This can be accomplished by using small diameter (e.g., 2 mm I.D.) capillary tubes to drain the test solution from spitter boxes into the replicate test chambers. The use of a self-starting siphon to gradually lower (i.e., less than or equal to 1 min.) the water level approximately 2 cm in the test chamber is recommended. A minimum water depth of 5 cm should be maintained in the cups. Although it may be satisfactory, a rocker-arm type apparatus has not yet been used with silversides.

(v) *Other equipment required.* (A) An apparatus for removing undesirable organisms, particulate matter and air bubbles.

(B) An apparatus for aerating water.

(C) A suitable magnifying viewer for examination of minnow embryos.

(D) A suitable apparatus for the precise measurement of growth of the fish, including both length (e.g., with metric or ruler caliper or photographic equipment) and weight.

(E) Facilities for providing a continuous supply of live brine shrimp nauplii (*Artemia salina*).

(F) For silversides, facilities for providing a supply of rotifers (*Brachionus plicatilis*) for approximately 11 days.

(G) Facilities (or access to facilities) for performing the required water chemistry analyses.

(vi) *Cleaning of equipment.* (A) Test substance delivery systems and test chambers should be cleaned before use. Test chambers should be cleaned during the test as needed to maintain the dissolved oxygen concentration, and to prevent clogging of the embryo cup screens and narrow flow passages.

(B) Debris can be removed with a rubber bulb and large pipette or by siphoning with a glass tube attached to a flexible hose. Debris should be run into a bucket light enough to observe that no live fish are accidentally discarded.

(vii) *Dilution water*—(A) *General.* (*1*) A constant supply of acceptable dilution water should be available for use throughout the test. Dilution water shall be of a minimum quality such that the test species selected will survive in it for the duration of testing without showing signs of stress (e.g., loss of pigmentation, disorientation, poor response to external stimuli, excessive mucous secretion, lethargy, lack of feeding, or other unusual behavior). A better criterion for an acceptable dilution water for tests on early life stages should be such that the species selected for testing will survive, grow, and reproduce satisfactorily in it.

(*2*) The concentration of dissolved oxygen in the dilution water (fresh or salt) shall be between 90 percent and 100 percent saturation. When necessary, dilution water should be aerated by means of airstones, surface aerators, or screen tubes before the introduction of the test substance.

(*3*) Water that is contaminated with undesirable microorganisms (e.g., fish pathogens) shall not be used. If such contamination is suspected, the water should be passed through a properly maintained ultraviolet sterilizer equipped with an intensity meter before use. Efficacy of the sterilizer can be determined by using standard plate count methods.

(B) *Freshwater.* (*1*) Natural water (clean surface or ground water) is preferred, however, dechlorinated tap water may be used as a last resort. Reconstituted freshwater is not recommended as a practical dilution water for the early life stage toxicity test because of the large volume of water required.

(*2*) Particulate and dissolved substance concentrations should be measured at least twice a year and should meet the following specifications:

| Substance | Concentration maximum |
|---|---|
| Particulate matter | <20 mg/liter. |
| Total organic carbon (TOC) | <2 mg/liter. |
| Chemical oxygen demand (COD) | <5 mg/liter. |
| Un-ionized ammonia | <1 µg/liter. |
| Residual chlorine | <1 µg/liter. |
| Total organophosphorus pesticides | <50 ng/liter. |
| Total organochlorine pesticides plus polychlorinated biphenyls (PCBs). | <50 ng/liter. |
| Total organic chlorine | <25 ng/liter. |

(*3*) During any one month, freshwater dilution water should not vary more than 10 percent from the respective monthly averages of hardness, alkalinity and specific conductance; the monthly pH range should be less than 0.4 pH units.

(C) *Saltwater.* (*1*) Marine dilution water is considered to be of constant quality if the minimum salinity is greater than 15‰ and the weekly range of the salinity is less than 15‰. The monthly range of pH shall be less than 0.8 pH units. Saltwater shall be filtered to remove larval predators. A pore size of <20 micrometers (um) is recommended. For silversides, the recommended salinity is 20 ppt and shall be maintained between 15 and 25 ppt throughout testing.

(*2*) Artificial sea salts may be added to natural seawater during periods of low salinity to maintain salinity above 15‰.

(3) *Test parameters*—(i) *Dissolved oxygen concentration.* It is recommended that the dissolved oxygen concentration be maintained between 90 and 100 percent saturation; but it shall be no less than 75 percent saturation at all times for both minnow species and between 90 and 100 percent saturation for the trout species in all test chambers. Dilution water in the head box may be aerated, but the test solution itself shall not be aerated.

(ii) *Loading and flow rate.* (A) The loading in test chambers should not exceed 0.1 grams of fish per liter of test solution passing through the test chamber in 24 hours. The flow rate to each chamber should be a minimum of 6 tank volumes per 24 hours. During a test, the flow rates should not vary more than 10 percent from any one test chamber to any other.

(B) A lower loading or higher flow rate or both shall be used if necessary to meet the following three criteria at all times during the test in each chamber containing live test organisms:

(*1*) The concentration of dissolved oxygen shall not fall below 75 percent saturation for the fathead and sheepshead minnows and 90 percent for the rainbow and brook trout;

(*2*) The concentration of un-ionized ammonia should not exceed 1 µg/1; and

(*3*) The concentration of toxicant should not be lowered (i.e., caused by uptake by the test organisms and/or materials on the sides and bottoms of the chambers) more than 20 percent of the mean measured concentration.

(iii) *Temperature.* (A) The recommended test temperatures are:

(*1*) Fathead minnow——25 °C for all life stages.

(*2*) Sheepshead minnow——30 °C for all life stages.

(*3*) Rainbow and brook trout——10 °C for embryos. 12 °C for fry and alevins.

(*4*) Atlantic and tidewater silversides——25 °C for all life stages.

(B) Excursions from the test temperature shall be no greater than ±2.0 °C. It is recommended that the test system be equipped with an automatic alarm system to alert staff of instantaneous temperature changes in excess of 2 °C. If the water is heated (i.e., for minnow species), precautions should be taken to ensure that supersaturation of dissolved gases is avoided. Temperatures shall be recorded in all test chambers at the beginning of the test and weekly thereafter. The temperature shall be recorded at least hourly in one test chamber throughout the test.

(iv) *Light.* (A) Brook and rainbow trout embryos shall be maintained in darkness or very low light intensity through one week post-hatch, at which time a 14-hour light and 10-hour dark photoperiod shall be provided.

(B) For fathead and sheepshead minnows, a 16-hour light and 8-hour dark (or 12:12) photoperiod shall be used throughout the test period.

(C) For silversides, a 14-hour light and 10-hour dark photoperiod shall be used throughout the test period.

(D) A 15-minute to 30-minute transition period between light and dark is optional.

(E) Light intensities ranging from 30 to 100 lumens at the water surface shall be provided; the intensity selected should be duplicated as closely as possible for all test chambers.

(e) *Reporting.* A report of the results of an early life stage toxicity test shall include the following:

(1) Name of test, sponsor, investigator, laboratory, and dates of test duration.

(2) Detailed description of the test substance including its source, lot number, composition (identity and concentration of major ingredients and major impurities), known physical and chemical properties, and any carriers (solvents) or other additives used.

(3) The source of the dilution water, its chemical characteristics, and a description of any pretreatment.

(4) Detailed information about the test organisms including scientific name and how verified and source history, observed diseases, treatments, acclimation procedure, and concentration of any contaminants and the method of measurement.

(5) A description of the experimental design and the test chambers, the depth and volume of the solution in the chambers, the way the test was begun, the number of organisms per treatment, the number of replicates, the loading, the lighting, a description of the test substance delivery system, and the flow rate as volume additions per 24 hours.

(6) Detailed information on feeding of fish during the toxicity test, including type of food used, its source, feeding frequency and results of analysis (i.e., concentrations) for contaminants.

(7) Number of embryos hatched, number of healthy embryos, time to hatch, mortality of embryos and fry, measurements of growth (weight and length), incidence of pathological or histological effects and observations of other effects or clinical signs, number of healthy fish at end of test.

(8) Number of organisms that died or showed an effect in the control and the results of analysis for concentration(s) of any contaminant in the control(s) should mortality occur.

(9) Methods used for, and the results of (with standard deviation), all chem-

ical analyses of water quality and test substance concentration, including validation studies and reagent blanks; the average and range of the test temperature(s).

(10) Anything unusual about the test, any deviation from these procedures, and any other relevant information.

(11) A description of any abnormal effects and the number of fish which were affected during each period between observations in each chamber, and the average concentration of test substance in each test chamber.

(12) Reference to the raw data location.

[50 FR 39321, Sept. 27, 1985, as amended at 52 FR 19064, May 20, 1987]

### § 797.1800 Oyster acute toxicity test.

(a) *Purpose.* This guideline will be used in developing data on the acute toxicity of chemical substances and mixtures ("chemicals") subject to environmental effects test regulations under the Toxic Substances Control Act (TSCA) (Pub. L. 94-469, 90 Stat. 2003, 15 U.S.C. 2601 *et. seq.*). This guideline prescribes tests to be used to develop data on the acute toxicity of chemicals to Eastern oysters, *Crassostrea virginica* (Gmelin). The United States Environmental Protection Agency (U.S. EPA) will use data from these tests in assessing the hazard of a chemical to the environment.

(b) *Definitions.* The definitions in section 3 of the Toxic Substances Control Act (TSCA) and in Part 792—*Good Laboratory Practice Standards* of this chapter are applicable to this test guideline. The following definitions also apply:

(1) "Acute toxicity" is the discernible adverse effects induced in an organism within a short period of time (days) of exposure to a chemical. For aquatic animals this usually refers to continuous exposure to the chemical in water for a period of up to four days. The effects (lethal or sublethal) occurring may usually be observed within the period of exposure with aquatic organisms. In this test guideline, shell deposition is used as the measure of toxicity.

(2) "$EC_{50}$" is that experimentally derived concentration of a chemical in water that is calculated to induce shell deposition 50 percent less than that of the controls in a test batch of organisms during continuous exposure within a particular exposure period which should be stated.

(3) "Shell deposition" is the measured length of shell growth that occurs between the time the shell is ground at test initiation and test termination 96 hours later.

(4) "Umbo" means the narrow end (apex) of the oyster shell.

(5) "Valve height" means the greatest linear dimension of the oyster as measured from the umbo to the ventral edge of the valves (the farthest distance from the umbo).

(c) *Test procedures*—(1) *Summary of the test.* (i) The water solubility and the vapor pressure of the test chemical should be known. Prior to testing, the structural formula of the test chemical, its purity, stability in water and light, n-octanol/water partition coefficient, and $pK_a$ values should be known prior to testing. The results of a biodegradability test and the method of analysis for the quantification of the chemical in water should also be known.

(ii) For chemicals with limited solubility under the test conditions, it may not be possible to determine an $EC_{50}$. If it is observed that the stability or homogeneity of the test chemical cannot be maintained, then care should be taken in the interpretation of the results and a note made that these results may not be reproducible.

(iii) Test chambers are filled with appropriate volumes of dilution water. The flow of dilution water through each chamber is adjusted to the rate desired. The test chemical is introduced into each test chamber and the flow-rate adjusted to establish and maintain the desired concentration in each test chamber. Test oysters which have been acclimated and prepared by grinding away a portion of the shell periphery are randomly introduced into the test and control chambers. Oysters in the test and control chambers are observed daily during the test for evidence of feeding or unusual conditions, such as shell gaping, excessive mucus production or formation of fungal growths in the test chambers.

The observations are recorded and dead oysters removed. At the end of 96 hours the increments of new shell growth are measured in all oysters. The concentration-response curve and $EC_{50}$ value for the test chemical are developed from these data.

(2) [Reserved]

(3) *Range-finding test.* A range-finding test should be conducted to establish test chemical concentrations for the definitive test. The test is conducted in the same way as the definitive test except a widely spaced chemical concentration series (i.e., log-interval) is used.

(4) *Definitive test.* (i) Oysters which meet condition criteria (age, size, reproductive status, health) and which have been acclimated to test conditions should have approximately 3 to 5 mm of the shell periphery, at the rounded (ventral) end, ground away with a small electric disc grinder or other appropriate device, taking care to uniformly remove the shell rim to produce a smooth, rounded, blunt profile. The oyster's valves should be held together tightly during grinding to avoid vibrating the shell and injuring the adductor muscle. Oysters of which so much of the shell rim has been removed that an opening into the shell cavity is visible shall not be used.

(ii) It is desirable to have shell growth values for the low and high concentrations relatively close to, but different from, 0 and 100 percent. Therefore, the range of concentrations to which the oysters are exposed should be such that in 96 hours relative to the controls, very little shell growth occurs in oysters exposed to the highest concentration and shell growth is slightly less than controls at the lowest concentration. Oysters in the remaining concentrations should have increments of shell growth, such that ideally, the concentration producing 50 percent shell growth relative to the growth is bracketed with at least one concentration above and one below it.

(iii) The test should be carried out without adjustment of pH unless there is evidence of marked change in the pH of the solution. Then it is advised that the test be repeated with pH adjustment to that of the dilution water and the results reported.

(iv) The test begins when at least 20 prepared oysters are placed in each of the test chambers containing the appropriate concentrations of test substance and controls. The steady-state flows and test chemical concentrations shall be documented. At least 5 test chemical concentrations shall be used. The dilution factor between concentrations shall not exceed 1.8.

(v) Test oysters shall be impartially distributed among test chambers in such a manner that test results show no significant bias from the distributions. The oysters should be spread out equidistantly from one another so that the entire test chamber is used. The oysters should also be placed with the left (cupped) valve down and the open, unhinged ends all oriented in the same direction facing the incoming flow of test solution.

(vi) The oysters are inspected at least after 24, 48, 72 and 96 hours. Oysters are considered dead if touching of the gaping shell produces no reaction. Dead oysters are removed when observed and mortalities are recorded. Observations at 3 hours and 6 hours are also desirable.

(vii) Shell growth is the primary criterion used in this test guideline to evaluate the toxicity of the test chemical. Shell growth increments in all oysters shall be measured after 96 hours exposure. Record the length of the longest "finger" of new shell growth to the nearest 0.5 mm. Oysters should be handled very gently at this stage to prevent damage to the new shell growth.

(viii) Records shall be kept of visible abnormalities such as loss of feeding activity (failure to deposit feces), excessive mucus production (stringy material floating suspended from oysters), spawning or appearance of shell (closure or gaping).

(ix) The criteria for a valid definitive test are:

(A) The mortality in the controls shall not exceed 10 percent at the end of the test.

(B) The dissolved oxygen concentration shall be at least 60 percent of air saturation throughout the test.

(C) If evidence of spawning is observed, the test shall be repeated.

(D) There shall be evidence that the concentration of the substance being tested has been satisfactorily maintained over the test period. The concentration of the test substance should be measured:

(*1*) In each chamber at 0-hour;

(*2*) In each chamber at 96-hours; and

(*3*) In at least one appropriate chamber whenever a malfunction is detected in any part of the test chemical delivery system.

(E) Dissolved oxygen, temperature, salinity, and pH measurements shall be made at the beginning and end of the test in each chamber.

(5) *Test results.* (i) At the end of the test, appropriate statistical analysis should be conducted on the oyster shell deposition test data. The probit transformation should then be applied to the response variable and then regressed, using least squares regression, on dose or log-dose. An F Test for linearity should be conducted to determine whether the chosen regression technique adequately describes the experimental data.

(ii) Calculate the ratio of the mean shell growth for each group of test oysters (exposed to each of the test chemical concentrations) to the mean shell growth of the group of control oysters. From these data the concentration-response curve is drawn and an $EC_{50}$ along with the 95 percent confidence limits on the value are determined from the curves. The mean measured concentration of test chemical should be used to calculate the $EC_{50}$ and to plot the concentration-response curve.

(6) [Reserved]

(d) *Test conditions*—(1) *Test species*—(i) *Selection.* (A) The Eastern oyster, *Crassostrea virginica*, shall be used as the test organism.

(B) Oysters used in the same test shall be 30 to 50 mm in valve height and should be as similar in age and/or size as possible to reduce variability. The standard deviation of the valve height should be less than 20 percent of the mean.

(C) Oysters used in the same test shall be from the same source and from the same holding and acclimation tank(s).

(D) Oysters shall be in a prespawn condition of gonadal development prior to and during the test as determined by direct or histological observation of the gonadal tissue for the presence of gametes.

(ii) *Acquisition.* Oysters may be cultured in the laboratory, purchased from culture facilities or commercial harvesters, or collected from a natural population in an unpolluted area free from epizootic disease.

(iii) *Acclimation.* (A) Oysters should be attended to immediately upon arrival. Oyster shells should be brushed clean of fouling organisms and the transfer of the oysters to the holding water should be gradual to reduce stress caused by differences in water quality characteristics and temperature. Oysters shall be held for at least 12 to 15 days before testing. All oysters shall be maintained in dilution water at the test temperature for at least 2 days before they are used.

(B) During holding, the oysters should not be crowded, and the dissolved oxygen concentration shall be above 60-percent saturation. The temperature of the holding water shall be the same as that used for testing. Holding tanks should be kept clean and free of debris. Cultured algae may be added to dilution water sparingly, as necessary to support life and growth and such that test results are not affected as confirmed by previous testing.

(C) Oysters should be handled as little as possible. When handling is necessary, it should be done as gently, carefully, and quickly as possible.

(D) A batch of oysters is acceptable for testing if the percentage mortality over the 7-day period prior to testing is less than 5 percent. If the mortality is between 5 and 10 percent, acclimation should continue for 7 additional days. If the mortality is greater than 10 percent, the entire batch of oysters shall be rejected. Oysters which appear diseased or otherwise stressed or which have cracked, chipped, bored, or gaping shells shall not be used. Oysters infested with mudworms (*Polydora sp.*) or boring sponges (*Cilona cellata*) should not be used.

(2) *Test facilities*—(i) *Apparatus.* (A) In addition to normal laboratory equipment, an oxygen meter, equipment for delivering the test chemical, adequate apparatus for temperature control, and test tanks made of chemically inert material are needed.

(B) Constant conditions in the test facilities should be maintained as much as possible throughout the test. The preparation and storage of the test material, the holding of the oysters and all operations and tests should be carried out in an environment free from harmful concentrations of dust, vapors and gases and in such a way as to avoid cross-contamination. Any disturbances that may change the behavior of the oysters should be avoided.

(ii) *Dilution water.* A constant supply of good quality unfiltered seawater should be available throughout the holding, acclimation and testing periods. Natural seawater is recommended, although artificial seawater with food added may be used. In either case, to ensure each oyster is provided equal amounts of food, the water should come from a thoroughly mixed common source and shall be delivered at a flowrate of at least 1 and preferably 5 liters per hour per oyster. The flowrate should be ±10 percent of the nominal flow. A dilution water is acceptable if oysters will survive and grow normally for 14 days without exhibiting signs of stress; i.e., excessive mucus production (stringy material floating suspended from oysters), lack of feeding, shell gaping, poor shell closing in response to prodding, or excessive mortality. The dilution water shall have a salinity in excess of 12 parts per thousand, and should be similar to that in the environment from which the test oysters originated. A natural seawater should have a weekly range in salinity of less than 10 parts per thousand and a monthly range in pH of less than 0.8 unit. Artificial seawater salinity should not vary more than 2 parts per thousand nor more than 0.5 pH unit. Oysters shall be tested in dilution water from the same origin.

(3) *Test parameters*—(i) *Carriers.* Stock solutions of substances of low aqueous solubility may be prepared by ultrasonic dispersion or, if necessary, by use of organic solvents, emulsifiers or dispersants of low toxicity to oysters. When such carriers are used the control oysters shall be exposed to the same concentration of the carrier as that used in the highest concentration of the test substance. The concentration of such carriers should not exceed 0.1 ml/l.

(ii) *Dissolved oxygen.* The dissolved oxygen concentrations shall be at least 60 percent of the saturation value and should be recorded daily.

(iii) *Loading.* The loading rate should not crowd oysters and should permit adequate circulation of water while avoiding physical agitation of oysters by water current.

(iv) *Temperature.* The test temperature shall be 20 °C. Temporary fluctuations (less than 8 hours) within ±5 °C are permissible. Temperature should be recorded continuously.

(v) *pH.* The pH shall be measured at the beginning and end of the test in each test chamber.

(e) *Reporting.* In addition to the reporting requirements prescribed in Part 792—*Good Laboratory Practice Standards* of this chapter, the report shall contain the following:

(1) The source of the dilution water, the mean, standard deviation and range of the salinity, pH, temperature, and dissolved oxygen during the test period.

(2) A description of the test procedures used (e.g., the flow-through system, test chambers, chemical delivery system, aeration, etc.).

(3) Detailed information about the oysters used, including the age and/or size (i.e., height), source, history, method of confirmation of prespawn condition, acclimation procedures, and food used.

(4) The number of organisms tested, the loading rate, and the flowrate.

(5) The methods of preparation of stock and test solutions, and the test chemical concentrations used.

(6) The number of dead and live test organisms, the percentage of organisms that died, and the number that showed any abnormal effects in the control and in each test chamber at each observation period.

(7) The 96-hour shell growth measurements of each oyster; the mean, standard deviation and range of the measured shell growth at 96 hours of oysters in each concentration of test substance and control.

(8) The calculated 96-hour $EC_{50}$ and its 95 percent confidence limits and the statistical methods used to calculate these values.

(9) When observed, the 96-hour observed no-effect concentration (the highest concentration tested at which there were no mortalities, abnormal behavioral or physiological effects and at which shell growth did not differ from controls).

(10) A graph of the concentration-response curve based on the 96-hour chemical concentration and shell growth measurements upon which the $EC_{50}$ was calculated.

(11) Methods and data records of all chemical analyses of water quality parameters and test substance concentrations, including method validations and reagent blanks.

(12) Any incidents in the course of the test which might have influenced the results.

(13) A statement that the test was carried out in agreement with the prescriptions of the test guideline given above (otherwise a description of any deviations occurring).

[50 FR 39321, Sept. 27, 1985, as amended at 52 FR 19066, May 20, 1987; 52 FR 26150, July 13, 1987]

### § 797.1830 Oyster bioconcentration test.

(a) *Purpose.* This guideline is to be used for assessing the propensity of chemical substances to bioconcentrate in tissues of estuarine and marine molluscs. This guideline describes a bioconcentration test procedure for the continuous exposure of Eastern oysters (*Crassostrea virginica*) to a test substance in a flow-through system. The United States Environmental Protection Agency (U.S. EPA) will use data from this test in assessing the hazard a chemical may present to the environment.

(b) *Definitions.* The definitions in section 3 of the Toxic Substances Control Act (TSCA) and in Part 792—*Good Laboratory Practice Standards* of this chapter are applicable to this test guideline. The following definitions also apply:

(1) "Acclimation" is the physiological compensation by test organisms to new environmental conditions (e.g., temperature, salinity, pH).

(2) "Bioconcentration" is the net accumulation of a chemical directly from water into and onto aquatic organisms.

(3) "Bioconcentration factor (BCF)" is the quotient of the concentration of a test chemical in tissues of aquatic organisms at or over a discrete time period of exposure divided by the concentration of test chemical in the test water at or during the same time period.

(4) "Depuration" is the elimination of a test chemical from a test organism.

(5) "Depuration phase" is the portion of a bioconcentration test after the uptake phase during which the organisms are in flowing water to which no test chemical is added.

(6) "$EC_{50}$" is that experimentally derived concentration of a chemical in water that is calculated to induce shell deposition 50 percent less than that of the controls in a test batch of organisms during continuous exposure within a particular period of exposure (which should be stated).

(7) "Loading" is the ratio of the number of oysters to the volume (liters) of test solution passing through the test chamber per hour.

(8) "Steady-state" is the time period during which the amounts of test chemical being taken up and depurated by the test oysters are equal, i.e., equilibrium.

(9) "Steady-state bioconcentration factor" is the mean concentration of the test chemical in test organisms during steady-state divided by the mean concentration of the test chemical in the test solution during the same period.

(10) "Umbo" is the narrow end (apex) of the oyster shell.

(11) "Uptake" is the sorption of a test chemical into and onto aquatic organisms during exposure.

(12) "Uptake phase" is the initial portion of a bioconcentration test during which the organisms are exposed to the test solution.

(13) "Valve height" is the greatest linear dimension of the oyster as measured from the umbo to the ventral edge of the valves (the farthest distance from the umbo).

(c) *Test procedures*—(1) *Summary of the test.* Oysters are continuously exposed to a minimum of one constant, sublethal concentration of a test chemical under flow-through conditions for a maximum of 28 days. During this time, test solution and oysters are periodically sampled and analyzed using appropriate methods to quantify the test chemical concentration. If, prior to day 28, the tissue concentrations of the chemical sampled over three consecutive sampling periods have been shown to be statistically similar (i.e., steady-state has been reached), the uptake phase of the test is terminated, and the remaining oysters are transferred to untreated flowing water until 95 percent of the accumulated chemical residues have been eliminated, or for a maximum depuration period of 14 days. The mean test chemical concentration in the oysters at steady-state is divided by the mean test solution concentration at the same time to determine the bioconcentration factor (BCF). If steady-state is not reached during 28 days of uptake, the steady-state BCF should be calculated using non-linear parameter estimation methods.

(2) [Reserved]

(3) *Range-finding test.* The oyster acute toxicity test is used to determine the concentration levels to be used in the oyster bioconcentration test.

(4) *Definitive test.* (i) The following data on the test chemical shall be known prior to testing:

(A) Solubility in water.

(B) Stability in water.

(C) Octanol-water partition coefficient.

(D) Acute toxicity (e.g., propensity to inhibit shell deposition) to oysters.

(E) The validity, accuracy and minimum detection limits of selected analytical methods.

(ii) At least one or more concentrations shall be tested to assess the propensity of the compound to bioconcentrate. The concentrations selected should not stress or adversely affect the oysters and should be less than one-tenth the $EC_{50}$ determined in either the rangefinding or 96-hour definitive test under § 797.1800 of this chapter. The test concentration shall be less than the solubility limit of the test substance in water and should be close to the potential or expected environmental concentration. The limiting factor of how low one can test is based on the detection limits of the analytical methods. The concentration of the test material in the test solution should be at least 10 times greater than the detection limit in water.

(iii) If it is desirable to document that the potential to bioconcentrate is independent of the test chemical concentration, at least two concentrations shall be tested which are at least a factor of 10 apart.

(iv) To determine the duration of this test, an estimation of the uptake phase shall be made prior to testing based upon the water solubility or octanol-water partition coefficient of the test chemical. This estimate shall also be used to designate a sampling schedule.

(v) The following criteria shall be met for a valid test:

(A) If it is observed that the stability or homogeneity of the test chemical cannot be maintained, then care should be taken in the interpretation of the results and a note shall be made that these results may not be reproducible.

(B) The mortality in the controls shall not exceed 10 percent at the end of the test.

(C) The dissolved oxygen concentration shall be $>$ 60 percent of saturation throughout the test.

(D) There shall be evidence that the concentration of the chemical being tested has been satisfactorily maintained over the test period.

(E) If evidence of spawning is observed, the test shall be repeated.

(F) Temperature variations from 20 °C should be held to a minimum.

(vi) The following methodology shall be followed:

(A) The test shall not be started until the test chemical delivery system has been observed to be functioning properly and the test chemical concentrations have equilibrated (i.e., the concentration does not vary more than

20 percent). Analyses of two sets of test solution samples taken prior to test initiation should document this equilibrium. At initiation (time 0), test solution samples shall be collected immediately prior to the addition of oysters to the test chambers.

(B) The appropriate number of oysters should be brushed clean and shall be impartially distributed among test chambers in such a manner that test results show no significant bias from the distributions. The oysters should be spread out equidistant from one another and placed with the left (cupped) valve down and the unhinged ends (opposite from umbo) all oriented in the same direction facing the incoming flow.

(C) Oysters shall be exposed to the test chemical during the uptake phase until steady state has been reached or for a maximum of 28 days. The uptake phase shall continue for at least 4 days. Then the remaining oysters shall be transferred to untreated flowing water and sampled periodically to determine if depuration of the test chemical occurs. Every test shall include a control consisting of the same dilution water, conditions, procedures, and oysters from the same group used in the test, except that none of the test chemical is added. If a carrier is present in the test chamber, a separate carrier control is required.

(D) Oysters shall be observed (and data recorded) at least daily for feeding activity (deposition of feces) or any unusual conditions such as excessive mucus production (stringy material floating suspended from oysters), spawning, or appearance of shell (closure or gaping). If gaping is noted, the oyster(s) should be prodded. Oysters which fail to make any shell movements when prodded are to be considered dead, and shall be removed promptly with as little disturbance as possible to the test chamber(s) and remaining live oysters.

(E) For oysters sampled, careful examination of all the tissues should be made at the time of shucking for any unusual conditions, such as a watery appearance or differences in color from the controls.

(F) Observations on compound solubility shall also be recorded. These include the appearance of surface slicks, precipitates, or material adsorbing to the test chamber.

(vii) *Sampling.* (A) At each of the designated sampling times, triplicate water samples and enough oysters shall be collected from the test chamber(s) to allow for tissue analyses of at least four oysters. The concentration of test chemical shall be determined in a minimum of four oysters analyzed individually at each sampling period. If individual analysis is not possible, due to limitations of the sensitivity of the analytical methods, then pairs, triplicates or more oysters may be pooled to constitute a sample for measurement. A similar number of control oysters shall also be collected at each sample point, but only those collected at the first sampling period and weekly thereafter, shall be analyzed. Triplicate control water samples shall be collected at the time of test initiation and weekly thereafter. Test solution samples shall be removed from the approximate center of the water column.

(B) At each sampling period the appropriate numbers of oysters are removed and treated as follows:

(*1*) The valve height of each oyster shall be measured.

(*2*) Oysters should be shucked as soon as practical after removal and should never be refrigerated or frozen in the shell. The shell should be opened at the hinge, the adductor muscle severed and the top valve removed. The remaining adductor muscle should be severed where it attaches to the lower valve and the entire oyster removed.

(*3*) The shucked oysters shall then be drained 3 minutes, blotted dry, weighed and analyzed immediately for the test chemical. If analyses are delayed, the shucked oysters shall be wrapped individually in aluminum foil (for organic analysis) or placed in plastic or glass containers (for metal analysis) and frozen.

(C) If a radiolabeled test compound is used, a sufficient number of oysters shall also be sampled at termination to permit identification and quantitation of any major (greater than 10 percent of parent) metabolites present. It is crucial to determine how much of the activity present in the oyster is directly attributable to the parent compound, and to correct the bioconcentration factor appropriately.

(5) *Test results* (i) Steady-state has been reached when the mean concentrations of test chemical in whole oyster tissue for three consecutive sampling periods are statistically similar (F test, $P=0.05$). A BCF is then calculated by dividing the mean tissue residue concentration during steady-state by the mean test solution concentration during the same period. A 95 percent confidence interval should also be derived from the BCF. This should be done by calculating the mean oyster tissue concentration at steady-state ($X_o$) and its 97.5 percent confidence interval $X_o \pm t$ (S.E.) where t is the t statistic at $P=0.025$ and S.E. is the one standard error of the mean. This calculation would yield lower and upper confidence limits ($L_o$ and $U_o$). The same procedure should be used to calculate the mean and 97.5 percent confidence interval for the test solution concentrations at steady-state, $XS \pm t$ (S.E.), and the resulting upper and lower confidence limits ($L_s$ and $U_s$). The 95 percent confidence interval of the BCF would then be between $L_o/U_s$ and $U_o/L_s$. If steady-state was not reached during the maximum 28-day uptake period, the maximum BCF should be calculated using the mean tissue concentration from that and the previous sampling day. An uptake rate constant should then be calculated using appropriate techniques. This rate constant is used to estimate the steady-state BCF and the time to steady-state.

(ii) If 95 percent elimination has not been observed after 14 days depuration then a depuration rate constant shall also be calculated. This rate constant should be based on the elimination of the parent compound.

(iii) Oysters used in the same test should be 30 to 50 millimeters in valve height and should be as similar in age and/or size as possible to reduce variability. The standard deviation of the height should be less than 20 percent of the mean ($N=30$).

(6) *Analytical measurements.* (i) All samples should be analyzed using USEPA methods and guidelines whenever feasible. The specific methodology used shall be validated before the test is initiated. The accuracy of the method should be measured by the method of known additions. This involves adding a known amount of the test chemical to three water samples taken from an aquarium containing dilution water and a number of oysters equal to that to be used in the test. The nominal concentration of these samples shall be the same as the concentration to be used in the test. Samples taken on two separate days shall be analyzed. The accuracy and precision of the analytical method shall be checked using reference or split samples or suitable corroborative methods of analysis. The accuracy of standard solutions should be checked against other standard solutions whenever possible.

(ii) An analytical method should not be used if likely degradation products of the test chemical, such as hydrolysis and oxidation products, give positive or negative interferences, unless it is shown that such degradation products are not present in the test chambers during the test. Atomic absorption spectrophotometric methods for metal and gas chromatographic methods for organic compounds are preferable to colorimetric methods.

(iii) In addition to analyzing samples of test solution at least one reagent blank shall also be analyzed when a reagent is used in the analysis.

(iv) When radiolabelled test compounds are used, total radioactivity shall be measured in all samples. At the end of the uptake phase, water and tissue samples shall be analyzed using appropriate methodology to identify and estimate the amount of any major (at least 10 percent of the parent compound) degradation products or metabolites that may be present.

(d) *Test conditions*—(1) *Test species.* (i) The Eastern oyster, *Crassostrea virginica*, shall be used as the test organism.

(ii) Oysters used in the same test should be 30 to 50 mm in valve height and should be as similar in age and/or size as possible to reduce variability. The standard deviation of the valve height shall be less than 20 percent of the mean.

(iii) Oysters used in the same test shall be from the same source and from the same holding and acclimation tank(s).

(iv) Oysters shall be in a prespawn condition of gonadal development prior to and during the test as determined by direct or histological observation of the gonadal tissue for the presence of gametes.

(v) Oysters may be cultured in the laboratory, purchased from culture facilities or commercial harvesters, or collected from a natural population in an unpolluted area free from epizootic disease.

(vi) The holding and acclimation of the oysters should be as follows:

(A) Oysters should be attended to immediately upon arrival. Oyster shells should be brushed clean of fouling organisms, and the transfer of the oysters to the holding water should be gradual to reduce stress caused by differences in water quality characteristics and temperature. Oysters shall be held for at least 12 to 15 days before testing. All oysters shall be maintained in dilution water at the test temperature for at least 2 days before they are used.

(B) During holding, the oysters should not be crowded, and the dissolved oxygen concentration shall be above 60-percent saturation. The temperature of the holding waters shall be the same as that used for testing. Holding tanks should be kept clean and free of debris. Cultured algae may be added to dilution water sparingly, as necessary to support life and growth, such that test results are not affected, as confirmed by previous testing. Oysters should be handled as little as possible. When handling is necessary, it should be done as gently, carefully, and quickly as possible.

(C) A batch of oysters is acceptable for testing if the percentage mortality over the 7-day period prior to testing is less than 5 percent. If the mortality is between 5 and 10 percent, acclimation shall continue for 7 additional days. If the mortality is greater than 10 percent, the entire batch of oysters shall be rejected. Oysters which appear diseased or otherwise stressed or which have cracked, chipped, bared, or gaping shells shall not be used. Oysters infested with mudworms (*Polydora sp.*) or boring sponges (*Cilona cellata*) should not be used.

(2) *Facilities*—(i) *Apparatus*. (A) An oxygen meter, equipment for delivering the test chemical, adequate apparatus for temperature control, test tanks made of chemically inert material and other normal laboratory equipment are needed.

(B) Constant conditions in the test facilities should be maintained as much as possible throughout the test. The preparation and storage of the test material, the holding of the oysters and all operations and tests should be carried out in an environment free from harmful concentrations of dust, vapors and gases and in such a way as to avoid cross-contamination. Any disturbances that may change the behavior of the oysters should be avoided.

(ii) *Dilution water*. A constant supply of good quality unfiltered seawater should be available throughout the holding, acclimation, and testing periods. Natural seawater is recommended, although artificial seawater with food (algae) added may be used. In either case, to ensure each oyster is provided equal amounts of food, the water should come from a thoroughly mixed common source and should be delivered at a flow rate of at least one, and preferably 5 liters per hour per oyster. The flowrate should be ±10 percent of the nominal flow. A dilution water is acceptable if oysters will survive and grow normally over the period in which the test is conducted without exhibiting signs of stress, i.e., excessive mucus production (stringy material floating suspended from oysters), lack of feeding, shell gaping, poor shell closing in response to prodding, or excessive mortality. The dilution water should have a salinity in excess of 12 parts per thousand, and should be similiar to that in the environment from which the test oysters originated. A natural seawater should have a weekly range in salinity of less than 10 parts per thousand and a monthly range in pH of less than 0.8 units. Artificial seawater should not vary more than 2 parts per thousand nor more than 0.5 pH units. Oysters should be tested in dilution water from the same origin.

(3) *Test parameters*—(i) *Carriers.* Stock solutions of substances of low aqueous solubility may be prepared by ultrasonic dispersion or, if necessary, by use of organic solvents, emulsifiers or dispersants of low toxicity to oysters. When such carriers are used, the control oysters shall be exposed to the same concentration of the carrier as that used in the highest concentration of the test substance. The concentration of such carriers shall not exceed 0.1 ml/l.

(ii) *Dissolved oxygen.* This dissolved oxygen concentration shall be at least 60 percent of the air saturation value and should be measured weekly in each chamber.

(iii) *Loading.* The loading rate shall not crowd oysters and shall permit adequate circulation of water while avoiding physical agitation of oysters by water current.

(iv) *Temperature.* The test temperature shall be 20 °C. Temporary excursions (less than 8 hours) within ± 5 °C are permissible. Temperature should be recorded continually.

(v) *pH.* The pH shall be measured weekly in each test chamber.

(e) *Reporting.* In addition to the reporting requirements prescribed in Part 792—*Good Laboratory Practice Standards* of this chapter, the report shall contain the following:

(1) The source of the dilution water, the mean, standard deviation and range of the salinity, pH, temperature and dissolved oxygen during the test period.

(2) A description of the test procedures used (e.g., the flow-through system, test chambers, chemical delivery system, aeration, etc.).

(3) Detailed information about the oysters used, including age, and/or size (i.e., height), weight (blotted dry), source, history, method of confirmation of prespawn condition, acclimation procedures, and food used.

(4) The number of organisms tested, loading rate and flowrate.

(5) The methods of preparation of stock and test solutions and the test chemical concentrations used.

(6) The number of dead and live organisms, the percentage of oysters that died and the number that showed any abnormal effects in the control and in each test chamber at each observation period.

(7) Methods and data records of all chemical analyses of water quality parameters and test chemical concentrations, including method validations and reagent blanks.

(8) Description of sampling, sample storage (if required) and analytical methods of water and tissue analyses for the test chemical.

(9) The mean, standard deviation and range of the concentration of test chemical in the test solution and oyster tissue at each sampling period.

(10) The time to steady-state.

(11) The steady-state or maximum BCF and the 95 percent confidence limits.

(12) The time to 95 percent elimination of accumulated residues of the test chemical from test oysters.

(13) Any incidents in the course of the test which might have influenced the results.

(14) If the test was not done in accordance with the prescribed conditions and procedures, all deviations shall be described in full.

[50 FR 39321, Sept. 27, 1985, as amended at 52 FR 19067, May 20, 1987]

## § 797.1930 Mysid shrimp acute toxicity test.

(a) *Purpose.* This guideline is intended for use in developing data on the acute toxicity of chemical substances and mixtures ("chemicals") subject to environmental effects test regulations under the Toxic Substances Control Act (TSCA) (Pub. L. 94-469, 90 Stat. 2003, 15 U.S.C. 2601 *et seq.*). This guideline prescribes a test using mysid shrimp as test organisms to develop data on the acute toxicity of chemicals. The United States Environmental Protection Agency (EPA) will use data from these tests in assessing the hazard of a chemical to the aquatic environment.

(b) *Definitions.* The definitions in section 3 of the Toxic Substances Control Act (TSCA) and in Part 792—*Good Laboratory Practice Standards* of this chapter, apply to this test guideline. The following definitions also apply to this guideline.

(1) "Death" means the lack of reaction of a test organism to gentle prodding.

(2) "Flow-through" means a continuous or an intermittent passage of test solution or dilution water through a test chamber or a holding or acclimation tank, with no recycling.

(3) "$LC_{50}$" means that experimentally derived concentration of test substance that is calculated to kill 50 percent of a test population during continuous exposure over a specified period of time.

(4) "Loading" means the ratio of test organisms biomass (grams, wet weight) to the volume (liters) of test solution in a test chamber.

(5) "Retention chamber" means a structure within a flow-through test chamber which confines the test organisms, facilitating observation of test organisms and eliminating loss of organisms in outflow water.

(6) "Static system" means a test chamber in which the test solution is not renewed during the period of the test.

(c) *Test procedures*—(1) *Summary of the test.* In preparation for the test, test chambers are filled with appropriate volumes of dilution water. If a flow-through test is performed, the flow of dilution water through each chamber is adjusted to the rate desired. The test substance is introduced into each test chamber. In a flow-through test, the rate at which the test substance is added is adjusted to establish and maintain the desired concentration of test substance in each test chamber. The test is started by randomly introducing mysids acclimated in accordance with the test design into the test chambers. Mysids in the test chambers are observed periodically during the test, the dead mysids removed and the findings recorded. Dissolved oxygen concentration, pH, temperature, salinity, the concentration of test substance, and other water quality characteristics are measured at specified intervals in test chambers. Data collected during the test are used to develop concentration-response curves and $LC_{50}$ values for the test substance.

(2) [Reserved]

(3) *Range-finding test.* (i) A range-finding test should be conducted to determine:

(A) Which life stage (juvenile or young adult) is to be utilized in the definitive test.

(B) The test solution concentrations for the definitive test.

(ii) The mysids should be exposed to a series of widely spaced concentrations of test substance (e.g., 1, 10, 100 mg/l, etc.), usually under static conditions.

(iii) This test should be conducted with both newly hatched juvenile (< 24 hours old) and young adult (5 to 6 days old) mysids. For each age class (juvenile or young adult), a minimum of 10 mysids should be exposed to each concentration of test substance for up to 96 hours. The exposure period may be shortened if data suitable for the purpose of the range-finding test can be obtained in less time. The age class which is most sensitive to the test substance in the range-finding test shall be utilized in the definitive test. When no apparent difference in sensitivity of the two life stages is found, juveniles shall be utilized in the definitive test. No replicates are required, and nominal concentrations of the chemical are acceptable.

(4) *Definitive test.* (i) The purpose of the definitive test is to determine the concentration-response curves and the 48- and 96-hour $LC_{50}$ values with the minimum amount of testing beyond the range-finding test.

(ii) The definitive test shall be conducted on the mysid life stage (juveniles or young adults) which is most sensitive to the test substance being evaluated.

(iii) A minimum of 20 mysids per concentration shall be exposed to five or more concentrations of the chemical chosen in a geometric series in which the ratio is between 1.5 and 2.0 (e.g., 2, 4, 8, 16, 32, and 64 mg/l). An equal number of mysids shall be placed in two or more replicates. If solvents, solubilizing agents or emulsifiers have to be used, they shall be commonly used carriers and shall not possess a synergistic or antagonistic effect on the toxicity of the test substance. The concentration of solvent shall not exceed 0.1 ml/1. The concentration ranges shall be selected to determine the concentration-response curves and $LC_{50}$ values at 48 and 96 hours.

(iv) Every test shall include controls consisting of the same dilution water, conditions, procedures, and mysids from the same population or culture container, except that none of the chemical is added.

(v) The dissolved oxygen concentration temperature, salinity, and pH shall be measured at the beginning and end of the test in each chamber.

(vi) The test duration is 96 hours. The test is unacceptable if more than 10 percent of the control organisms die or exhibit abnormal behavior during the 96 hour test period. Each test chamber should be checked for dead mysids at 24, 48, 72, and 96 hours after the beginning of the test. Concentration-response curves and 24-, 48-, 72- and 96-hour $LC_{50}$ values should be determined along with their 95 percent confidence limits.

(vii) In addition to death, any abnormal behavior or appearance shall also be reported.

(viii) Test organisms shall be impartially distributed among test chambers in such a manner that test results show no significant bias from the distributions. In addition, test chambers within the testing area shall be positioned in a random manner or in a way in which appropriated statistical analyses can be used to determine the variation due to placement.

(ix) The concentration of the test substance in the chambers should be measured as often as is feasible during the test. At a minimum, during static tests the concentration of test substance shall be measured at each concentration at the beginning and at the end of the test. During the flow-through test, the concentration of test substance should be measured at the beginning and end of the test and in at least one appropriate chamber whenever a malfunction is detected in any part of the test substance delivery system. Equal aliquots of test solution may be removed from each replicate chamber and pooled for analysis. Among replicate test chambers of a treatment concentration, the measured concentration of the test substance should not vary more than 20 percent.

(5) [Reserved]

(6) *Analytical measurements*— (i) *Test chemical.* Deionized water should be used in making stock solutions of the test substance. Standard analytical methods should be used whenever available in performing the analyses. The analytical method used to measure the amount of test substance in a sample shall be validated before beginning the test by appropriate laboratory practices. An analytical method is not acceptable if likely degradation products of the test substance, such as hydrolysis and oxidation products, give positive or negative interferences which cannot be systematically identified and corrected mathematically.

(ii) *Numerical.* The number of dead mysids shall be counted during each definitive test. Appropriate statistical analyses should provide a goodness-of-fit determination for the concentration-response curves. A 48- and 96-hour $LC_{50}$ and corresponding 95 percent interval shall be calculated.

(d) *Test conditions*—(1) *Test species*—(i) *Selection.* (A) The mysid shrimp, ***Mysidopsis bahia***, is the organism specified for these tests. Either juvenile (<24 hours old) or young adult (5 to 6 days old) mysids are to be used to start the test.

(B) Mysids to be used in chronic toxicity tests should originate from laboratory cultures in order to ensure the individuals are of similar age and experimental history. Mysids used for establishing laboratory cultures may be purchased commercially or collected from appropriate natural areas. Because of similarities with other mysids species, taxonomic verification should be obtained from the commercial supplier by experienced laboratory personnel or by an outside expert.

(C) Mysids used in a particular test shall be of similar age and be of normal size and appearance for their age. Mysids shall not be used for a test if they exhibit abnormal behavior or if they have been used in a previous test, either in a treatment or in a control group.

(ii) *Acclimation.* (A) Any change in the temperature and chemistry of the dilution water used for holding or culturing the test organisms to those of the test shall be gradual. Within a 24-hour period, changes in water temperature shall not exceed 1 °C, while salinity changes shall not exceed 5 percent.

(B) During acclimation mysids should be maintained in facilities with background colors and light intensities similar to those of the testing areas.

(iii) *Care and handling.* Methods for the care and handling of mysids such as those described in paragraph (f)(1) of this section can be used during holding, culturing and testing periods.

(iv) *Feeding.* Mysids should be fed during testing. Any food utilized should support survival, growth and reproduction of the mysids. A recommended food is live *Artemia* spp. (48-hour-old nauplii).

(2) *Facilities*—(i) *Apparatus.* (A) Facilities which may be needed to perform this test include: (*1*) flow-through or recirculating tanks for holding and acclimating mysids; (*2*) a mechanism for controlling and maintaining the water temperature during the holding, acclimation and test periods; (*3*) apparatus for straining particulate matter, removing gas bubbles, or aerating the water, as necessary; and (*4*) an apparatus for providing a 14-hour light and 10-hour dark photoperiod with a 15 to 30 minute transition period. In addition, for flow-through tests, flow-through chambers and a test substance delivery system are required. Furthermore, it is recommended that mysids be held in retention chambers within test chambers to facilitate observations and eliminate loss of test organisms through outflow water. For static tests, suitable chambers for exposing test mysids to the test substance are required. Facilities should be well ventilated and free of fumes and disturbances that may affect the test organisms.

(B) Test chambers shall be loosely covered to reduce the loss of test solution or dilution water due to evaporation and to minimize the entry of dust or other particulates into the solutions.

(ii) *Cleaning.* Test substance delivery systems and test chambers shall be cleaned before each test following standard laboratory practices.

(iii) *Construction materials.* (A) Materials and equipment that contact test solutions should be chosen to minimize sorption of test chemicals from dilution water and should not contain substances that can be leached into aqueous solution in quantities that can affect test results.

(B) For use in the flow-through test, retention chambers utilized for confinement of test organisms can be constructed with netting material of appropriate mesh size.

(iv) *Dilution water.* (A) Natural or artificial seawater is acceptable as dilution water if mysids will survive and successfully reproduce in it for the duration of the holding, acclimating and testing periods without showing signs of stress, such as reduced growth and fecundity. Mysids shall be cultured and tested in dilution water from the same origin.

(B) Natural seawater shall be filtered through a filter with a pore size of <20 microns prior to use in a test.

(C) Artificial seawater can be prepared by adding commercially available formulations or by adding specific amounts of reagent-grade chemicals to deionized water. Deionized water with a conductivity less than 1 $\mu$ ohm/cm at 12 °C is acceptable for making artificial seawater. When deionized water is prepared from a ground or surface water source, conductivity and total organic carbon (or chemical oxygen demand) shall be measured on each batch.

(v) *Test substance delivery system.* In flow-through tests, proportional diluters, metering pumps, or other suitable systems should be used to deliver test substance to the test chambers. The system used shall be calibrated before each test. Calibration includes determining the flow rate through each chamber and the concentration of the test substance in each chamber. The general operation of the test substance delivery system should be checked twice daily during a test. The 24-hour flow through a test chamber shall be equal to at least 5 times the volume of the test chamber. During a test, the flow rates should not vary more than 10 percent among test chambers or across time.

(3) *Test parameters.* Environmental parameters of the water contained in test chambers shall be maintained as specified below:

(i) The test temperature shall be 25°C. Excursions from the test temperature shall be not greater than ± 2°C.

(ii) Dissolved oxygen concentration between 60 and 105 percent saturation. Aeration, if needed to achieve this level, shall be done before the addition of the test substance. All treatment and control chambers shall be given the same aeration treatment.

(iii) The number of mysids placed in a test solution shall not be so great as to affect results of the test. Loading shall not exceed 30 mysids per liter for a static test. Loading requirements for the flow-through test will vary depending on the flow rate of dilution water. The loading shall not cause the dissolved oxygen concentration to fall below the recommended levels.

(iv) Photoperiod of 14 hours light and 10 hours darkness, with a 15 to 30 minute transition period.

(v) Salinity of 20 parts per thousand ±3 percent.

(e) *Reporting.* The sponsor shall submit to the EPA all data developed during the test that are suggestive or predictive of acute toxicity and all concomitant toxicologic manifestations. In addition to the general reporting requirements prescribed in Part 792—*Good Laboratory Practice Standards* of this chapter, the reporting of test data shall include the following:

(1) The source of the dilution water, its chemical characteristics (e.g., salinity, pH, etc.) and a description of any pretreatment.

(2) Detailed information about the test organisms, including the scientific name and method of verification, age, source, history, abnormal behavior, acclimation procedures and food used.

(3) A description of the test chambers, the depth and volume of solution in the chamber, the way the test was begun (e.g., conditioning, test substance additions, etc.), the number of organisms per treatment, the number of replicates, the loading, the lighting, the test substance delivery system and the flow rate expressed as volume additions per 24 hours.

(4) The measured concentration of test substance in test chambers at the times designated.

(5) The number and percentage of organisms that died or showed any other adverse effects in the control and in each treatment at each observation period.

(6) Concentration-response curves shall be fitted to mortality data collected at 24, 48, 72, and 96 hours. A statistical test of goodness-of-fit shall be performed and the results reported.

(7) The 96-hour $LC_{50}$ and when sufficient data have been generated, the 24-, 48-, and 72-hour $LC_{50}$'s and the corresponding 95-percent confidence limits and the methods used to calculate the values. These calculations shall be made using the average measured concentration of the test substance.

(8) Methods and data records of all chemical analyses of water quality and test substance concentrations, including method validations and reagent blanks.

(9) The data records of the holding, acclimation and test temperature and salinity.

(f) *References.* For additional background information on this test guideline the following references should be consulted:

(1) U.S. Environmental Protection Agency, "Bioassay Procedures for the Ocean Disposal Permit Program," EPA Report No. 600-9-78-010 (Gulf Breeze, Florida, 1978).

(2) [Reserved]

[50 FR 39321, Sept. 27, 1985, as amended at 52 FR 19068, May 20, 1987; 52 FR 26150, July 13, 1987]

## § 797.1950 Mysid shrimp chronic toxicity test.

(a) *Purpose.* This guideline is intended for use in developing data on the chronic toxicity of chemical substances and mixtures ("chemicals") subject to environmental effects test regulations under the Toxic Substances Control Act (TSCA) (Pub. L. 94-469, 90 Stat. 2003, 15 U.S.C. 2601 *et seq.*). This guideline prescribes tests using mysids as test organisms to develop data on the chronic toxicity of chemicals. The United States Environmental Protection Agency (EPA) will use data from these tests in assessing the hazard of a chemical to the aquatic environment.

(b) *Definitions.* The definitions in section 3 of the Toxic Substances Control Act (TSCA) and in Part 792—*Good Laboratory Practice Standards* of this chapter apply to this test guideline. The following definitions also apply to this guideline:

(1) "Chronic toxicity test" means a method used to determine the concentration of a substance that produces an adverse effect from prolonged exposure of an organism to that substance. In this test, mortality, number of young per female and growth are used as measures of chronic toxicity.

(2) "Death" means the lack of reaction of a test organism to gentle prodding.

(3) "Flow-through" means a continuous or an intermittent passage of test solution or dilution water through a test chamber or a holding or acclimation tank, with no recycling.

(4) "G1 (Generation 1)" means those mysids which are used to begin the test, also referred to as adults; G2 (Generation 2) are the young produced by G1.

(5) "$LC_{50}$" means that experimentally derived concentration of test substance that is calculated to kill 50 percent of a test population during continuous exposure over a specified period of time.

(6) "Loading" means the ratio of test organism biomass (gram, wet weight) to the volume (liters) of test solution in a test chamber.

(7) "MATC" (Maximum Acceptable Toxicant Concentration) means the maximum concentration at which a chemical can be present and not be toxic to the test organism.

(8) "Retention chamber" means a structure within a flow-through test chamber which confines the test organisms, facilitating observation of test organisms and eliminating washout from test chambers.

(c) *Test procedures*—(1) *Summary of the test.* (i) In preparation for the test, the flow of test solution through each chamber is adjusted to the rate desired. The test substance is introduced into each test chamber. The rate at which the test substance is added is adjusted to establish and maintain the desired concentration of test substance in each test chamber. The test is started by randomly introducing mysids acclimated in accordance with the test design into retention chambers within the test and the control chambers. Mysids in the test and control chambers are observed periodically during the test, the dead mysids removed and the findings reported.

(ii) Dissolved oxygen concentration, pH, temperature, salinity, the concentration of test substance and other water quality characteristics are measured at specified intervals in selected test chambers.

(iii) Data collected during the test are used to develop a MATC (Maximum Acceptable Toxicant Concentration) and quantify effects on specific chronic parameters.

(2) [Reserved]

(3) *Range-finding test.* (i) A range-finding test should be conducted to establish test solution concentrations for the definitive test.

(ii) The mysids should be exposed to a series of widely spaced concentrations of the test substance (e.g., 1, 10, 100 mg/l), usually under static conditions.

(iii) A minimum of 10 mysids should be exposed to each concentration of test substance for a period of time which allows estimation of appropriate chronic test concentrations. No replicates are required and nominal concentrations of the chemical are acceptable.

(4) *Definitive test.* (i) The purpose of the definitive test is to determine concentration-response curves, $LC_{50}$ values, and effects of a chemical on growth and reproduction during chronic exposure.

(ii) A minimum of 40 mysids per concentration shall be exposed to four or more concentrations of the chemical chosen in a geometric series in which the ratio is between 1.5 and 2.0 (e.g., 2, 4, 8, 16, 32, and 64 mg/1). An equal number of mysids shall be placed in two or more replicates. If solvents, solubilizing agents or emulsifiers have to be used, they shall be commonly used carriers and shall not possess a synergistic or antagonistic effect on the toxicity of the test substance. The concentration of solvent should not exceed 0.1 ml/1. The concentration ranges should be selected to determine the concentration response curves, $LC_{50}$ values and MATC. Concentration of test substance in test solutions should be analyzed prior to use.

(iii) Every test should include controls consisting of the same dilution water, conditions, procedures and mysids from the same population or culture container, except that none of the chemical is added.

(iv) The dissolved oxygen concentration, temperature, salinity, and pH shall be measured weekly in each chamber.

(v) The test duration is 28 days. The test is unacceptable if more than 20 percent of the control organisms die, appear stressed or are diseased during the test. The number of dead mysids in each chamber shall be recorded on days 7, 14, 21, and 28 of the test. At the time when sexual characteristics are discernible in the mysids (approximately 10 to 12 days in controls; possible delays may occur in mysids exposed to test substances), the number of males and females (identified by ventral brood pouch) in each chamber shall be recorded. Body length (as measured by total midline body length, from the anterior tip of the carapace to the posterior margin of the uropod) shall be recorded for males and females at the time when sex can be determined simultaneously for all mysids in control and treatment groups. This time cannot be specified because of possible delays in sexual maturation of mysids exposed to test substances. A second observation of male and female body lengths shall be conducted on day 28 of the test. To reduce stress on the mysids, body lengths can be recorded by photography through a stereomicroscope with appropriate scaling information. As offspring are produced by the G1 mysids (approximately 13 to 16 days in controls), the young shall be counted and separated into retention chambers at the same test substance concentration as the chambers where they originated. If available prior to termination of the test, observations on the mortality, number of males and females and male and female body length shall be recorded for the G2 mysids. Concentration-response curves, $LC_{50}$ values and associated 95 percent confidence limits for the number of dead mysids (G1) shall be determined for days 7, 14, 21, and 28. An MATC shall be determined for the most sensitive test criteria measured (cumulative mortality of adult mysids, number of young per female, and body lengths of adult males and females).

(vi) In addition to death, any abnormal behavior or appearance shall also be reported.

(vii) Test organisms shall be impartially distributed among test chambers in such a manner that test results show no significant bias from the distributions. In addition, test chambers within the testing area shall be positioned in a random manner or in a way in which appropriate statistical analyses can be used to determined the variation due to placement.

(viii) The concentration of the test substance in the chambers should be measured as often as is feasible during the test. The concentration of test substance shall be measured:

(A) At each test concentration at the beginning of the test and on days 7, 14, 21, and 28; and

(B) In at least one appropriate chamber whenever a malfunction is detected in any part of the test substance delivery system.

Equal aliquots of test solutions may be removed from each test chamber and pooled for analysis. Among replicate test chambers of a treatment concentration, the measured concentration of the test substance should not vary more than 20 percent.

(5) [Reserved]

(6) *Analytical measurements*—(i) *Test chemical.* Deionized water should be used in making stock solutions of the test substance. Standard analytical methods should be employed whenever available in performing the analyses. The analytical method used to measure the amount of test substance in a sample shall be validated before beginning the test by appropri-

ate laboratory practices. An analytical method is not acceptable if likely degradation products of the test substance, such as hydrolysis and oxidation products, give positive or negative interferences which cannot be systematically identified and corrected mathematically.

(ii) *Numerical.* (A) The number of dead mysids, cumulative young per female, and body lengths of male and female mysids shall be recorded during each definitive test. Appropriate statistical analyses shall provide a goodness-of-fit determination for the day 7, 14, 21 and 28 adult (Gl) death concentration-response curves.

(B) A 7-, 14-, 21- and 28-day $LC_{50}$, based on adult (Gl) death, and corresponding 95 percent confidence intervals shall be calculated. Appropriate statistical tests (e.g., analysis of variance, mean separation test) should be used to test for significant chemical effects on chronic test criteria (cumulative mortality of adults, cumulative number of young per female and body lengths of adult male and females) on designated days. An MATC shall be calculated using these chronic tests criteria.

(d) *Test conditions*—(1) *Test species*—(i) *Selection.* (A) The mysid shrimp, *Mysidopsis bahia*, is the organism specified for these tests. Juvenile mysids, <24 hours old, are to be used to start the test.

(B) Mysids to be used in chronic toxicity tests should originate from laboratory cultures in order to ensure the individuals are of similar age and experimental history. Mysids used for establishing laboratory cultures may be purchased commercially or collected from appropriate natural areas. Because of similarities with other mysid species, taxonomic verification should be obtained from the commercial supplier, by experienced laboratory personnel, or by an outside expert.

(C) Mysids used in a particular test shall be of similar age and be of normal size and appearance for their age.

(D) Mysids shall not be used for a test if they exhibit abnormal behavior, or if they have been used in a previous test, either in a treatment or in a control group.

(ii) *Acclimation.* (A) Any change in the temperature and chemistry of the water used for holding or culturing the test organisms to those of the test should be gradual. Within a 24-hour period, changes in water temperature should not exceed 1 °C, while salinity changes should not exceed 5 percent.

(B) During acclimation mysids should be maintained in facilities with background colors and light intensities similar to those of the testing areas.

(iii) *Care and handling.* Methods for the care and handling of mysids such as those described in paragraph (f)(1) of this section can be used during holding, culturing and testing periods.

(iv) *Feeding.* Mysids should be fed during testing. Any food utilized should support survival, growth and reproduction of the mysids. A recommended food is live *Artemia* spp. nauplii (approximately 48 hours old).

(2) *Facilities*—(i) *Apparatus.* (A) Facilities which may be needed to perform this test include: (*1*) flow-through or recirculating tanks for holding and acclimating mysids; (*2*) a mechanism for controlling and maintaining the water temperature during the holding, acclimation and test periods; (*3*) apparatus for straining particulate matter, removing gas bubbles, or aerating the water, as necessary; and (*4*) an apparatus for providing a 14-hour light and 10-hour dark photoperiod with a 15- to 30-minute transition period. In addition, flow-through chambers and a test substance delivery system are required. It is recommended that mysids be held in retention chambers within test chambers to facilitate observations and eliminate loss through outflow water.

(B) Facilities should be well ventilated and free of fumes and disturbances that may affect test organisms.

(C) Test chambers shall be loosely covered to reduce the loss of test solution or dilution water due to evaporation and to minimize the entry of dust or other particulates into the solutions.

(ii) *Cleaning.* Test substance delivery systems and test chambers shall be cleaned before each use following standard laboratory practices.

(iii) *Construction materials.* (A) Materials and equipment that contact test solutions should be chosen to minimize sorption of test chemicals from the dilution water and should not contain substances that can be leached into aqueous solution in quantities that can affect the test results.

(B) Retention chambers utilized for confinement of test organisms can be constructed with netting material of appropriate mesh size.

(iv) *Dilution water.* (A) Natural or artificial seawater is acceptable as dilution water if mysids will survive and successfully reproduce in it for the duration of the holding, acclimating and testing periods without showing signs of stress, such as reduced growth and fecundity. Mysids shall be cultured and tested in dilution water from the same origin.

(B) Natural seawater shall be filtered through a filter with a pore size of >20 microns prior to use in a test.

(C) Artificial seawater can be prepared by adding commercially available formulations or by adding specific amounts of reagent-grade chemicals to deionized or glass-distilled water. Deionized water with a conductivity less than 1 μ ohm/cm at 12 °C is acceptable as the diluent for making artificial seawater. When deionized water is prepared from a ground or surface water source, conductivity and total organic carbon (or chemical oxygen demand) shall be measured on each batch.

(v) *Test substance delivery system.* Proportional diluters, metering pumps, or other suitable systems should be used to deliver test substance to the test chambers. The system used shall be calibrated before each test. Calibration includes determining the flow rate and the concentration of the test substance in each chamber. The general operation of the test substance delivery system should be checked twice daily during a test. The 24-hour flow rate through a chamber shall be equal to at least 5 times the volume of the chamber. The flow rates should not vary more than 10 percent among chambers or across time.

(3) *Test parameters.* Environmental parameters of the water contained in test chambers shall be maintained as specified below:

(i) The test temperature shall be 25°C. Excursions from the test temperature shall be no greater than ± 2°C.

(ii) Dissolved oxygen concentration between 60 and 105 percent saturation. Aeration, if needed to achieve this level, shall be done before the addition of the test substance. All treatment and control chambers shall be given the same aeration treatment.

(iii) The number of mysids placed in a test solution shall not be so great as to affect results of the test. Loading requirements for the test will vary depending on the flow rate of dilution water. The loading shall not cause the dissolved oxygen concentration to fall below the recommended levels.

(iv) Photoperiod of 14 hours light and 10 hours darkness, with a 15–30 minute transition period.

(v) Salinity of 20 parts per thousand ±3 percent.

(e) *Reporting.* The sponsor shall submit to the EPA all data developed by the test that are suggestive or predictive of chronic toxicity and all concomitant toxicologic manifestations. In addition to the general reporting requirements prescribed in Part 792—*Good Laboratory Practice Standards* of this chapter, the reporting of test data shall include the following:

(1) The source of the dilution water, its chemical characteristics (e.g., salinity, pH, etc.) and a description of any pretreatment.

(2) Detailed information about the test organisms, including the scientific name and method of verification, average length, age, source, history, observed diseases, treatments, acclimation procedures and food used.

(3) A description of the test chambers, the depth and volume of solution in the chamber, the way the test was begun (e.g., conditioning, test substance additions, etc.), the number of organisms per treatment, the number of replicates, the loading, the lighting, the test substance delivery system, and the flow rate expressed as volume additions per 24 hours.

(4) The measured concentration of test substance in test chambers at the times designated.

(5) The first time (day) that sexual characteristics can be observed in con-

trols and in each test substance concentration.

(6) The length of time for the appearance of the first brood for each concentration.

(7) The means (average of replicates) and respective 95 percent confidence intervals for:

(i) Body length of males and females at the first observation day (depending on time of sexual maturation) and on day 28.

(ii) Cumulative number of young produced per female on day 28.

(iii) Cumulative number of dead adults on day 7, 14, 21 and 28.

(iv) If available prior to test termination (day 28), effects on G2 mysids (number of males and females, body length of males and females and cumulative mortality).

(8) The MATC is calculated as the geometric mean between the lowest measured test substance concentration that had a significant ($P<0.05$) effect and the highest measured test substance concentration that had no significant ($P<0.05$) effect in the chronic test. The most sensitive of the test criteria for adult (G1) mysids (cumulative number of dead mysids, body lengths of males and females or the number of young per female) is used to calculate the MATC. The criterion selected for MATC computation is the one which exhibits an effect (a statistically significant difference between treatment and control groups; $P<0.05$) at the lowest test substance concentration for the shortest period of exposure. Appropriate statistical tests (analysis of variance, mean separation test) should be used to test for significant chemical effects. The statistical tests employed and the results of these tests shall be reported.

(9) Concentration-response curves shall be fitted to the cumulative number of adult dead for days 7, 14, 21, and 28. A statistical test of goodness-of-fit shall be performed and the results reported.

(10) An $LC_{50}$ value based on the number of dead adults with corresponding 95 percent confidence intervals for days 7, 14, 21 and 28. These calculations shall be made using the average measured concentration of the test substance.

(11) Methods and data records of all chemical analyses of water quality and test substance concentrations, including method validations and reagent blanks.

(12) The data records of the holding, acclimation and test temperature and salinity.

(f) *References.* For additional background information on this test guideline the following references should be consulted:

(1) U.S. Environmental Protection Agency, "Bioassay Procedures for the Ocean Disposal Permit Program," EPA Report No. 600/9-78-010 (Gulf Breeze, Florida, 1978).

(2) [Reserved]

[50 FR 39321, Sept. 27, 1985, as amended at 52 FR 19069, May 20, 1987]

## § 797.1970 Penaeid shrimp acute toxicity test.

(a) *Purpose.* This guideline is intended for use in developing data on the acute toxicity of chemical substances and mixtures ("chemicals") subject to environmental effects test regulations under the Toxic Substances Control Act (TSCA) (Pub. L. 94-469, 90 Stat. 2003, 15 U.S.C. 2601 *et seq.*). This guideline prescribes tests using penaeid shrimp as test organisms to develop data on the acute toxicity of chemicals. The United States Environmental Protection Agency (EPA) will use data from these tests in assessing the hazard of a chemical to the aquatic environment.

(b) *Definitions.* The definitions in section 3 of the Toxic Substances Control Act (TSCA), and in Part 792—*Good Laboratory Practice Standards* of this chapter apply to this test guideline. The following definitions also apply to this guideline:

(1) "Death" means the lack of reaction of a test organism to gentle prodding.

(2) "Flow-through" means a continuous passage of test solution or dilution water through a test chamber, holding or acclimation tank with no recycling.

(3) "$LC_{50}$" means that experimentally derived concentration of test substance that is calculated to have killed 50 percent of a test population during

continuous exposure over a specified period of time.

(4) "Loading" means the ratio of test organism biomass (grams, wet weight) to the volume (liters) of test solution in a test chamber.

(c) *Test procedures*—(1) *Summary of the test.* Prior to testing, the bottoms of the test chambers are covered with 2 to 3 cm of sand and then filled with appropriate volumes of dilution water. The flow is adjusted to the rate desired to achieve loading requirements. Penaeid shrimp are introduced into the test chambers according to the experimental design. The shrimp are maintained in the test chambers for a period of 3 to 7 days prior to the beginning of the test. The test begins when the test substance is introduced into the test chambers. The rate of flow is adjusted to maintain the desired test substance concentration in each chamber. The shrimp are observed during the test; dead shrimp are counted, removed, and the findings recorded. Dissolved oxygen concentration, pH, temperature, salinity, test substance concentration and other water quality characteristics are measured at specified intervals in selected test chambers. Data collected during the test are used to develop concentration-response curves and $LC_{50}$ values for the test substance.

(2) [Reserved]

(3) *Range-finding test.* (i) A range-finding test should be conducted to determine the test substance concentrations to be used for the definitive test.

(ii) The shrimp should be exposed to a series of widely spaced concentrations of test substance (e.g., 1, 10, 100 mg/l, etc.).

(iii) A minimum of five penaeid shrimp should be exposed to each concentration of test substance for up to 96 hours. No replicates are required and nominal concentrations of the chemical are acceptable.

(4) *Definitive test.* (i) The purpose of the definitive test is to determine the concentration-response curves and the 48- and 96-hour $LC_{50}$ values with the minimum amount of testing beyond the range-finding test.

(ii) A minimum of 20 shrimp per concentration should be exposed to five or more concentrations of the chemical chosen in a geometric series in which the ratio is between 1.5 and 2.0 (e.g., 2, 4, 8, 16, 32 and 64 mg/l). An equal number of shrimp should be placed in two or more replicates. If solvents, solubilizing agents or emulsifiers have to be used, they should be commonly used carriers and should not possess a synergistic or antagonistic effect on the toxicity of the test substance. The concentration of solvent should not exceed 0.1 ml/l. The concentration ranges should be selected to determine the requested concentration-response curves and $LC_{50}$ values. The concentration of test substance in test solutions should be analyzed for chemical concentration prior to use and at designated times.

(iii) Every test should include controls consisting of the same dilution water, conditions, procedures and shrimp from the same population or culture container, except that none of the chemical is added.

(iv) The dissolved oxygen concentration, temperature, salinity and pH should be measured at the beginning of the test and at 24, 48, 72, and 96 hours in each test chamber.

(v) The test duration is 96 hours. The test is unacceptable if more than 10 percent of the control organisms die or appear to be stressed or diseased during the 96 hour test period. Each test chamber should be checked for dead shrimp at 3, 6, 12, 24, 48, 72, and 96 hours after the beginning of the test. Concentration-response curves and 48- and 96-hour $LC_{50}$ values should be determined along with their 95 percent confidence limits.

(vi) In addition to death, any abnormal behavior or appearance should also be reported.

(vii) Distribution of shrimp among test chambers should be randomized. In addition, test chambers within the testing area should be positioned in a random manner or in a way in which appropriate statistical analyses can be used to determine the variation due to placement.

(viii) The concentration of dissolved test substance (that which passes through a 0.45 micron filter) in the test chambers should be measured as often as is feasible during the test.

The concentration of test substance should be measured:

(A) In each chamber at the beginning of the test and at 48 and 96 hours after the start of the test.

(B) In at least one chamber containing the next to the lowest test substance concentration at least once every 24 hours during the test.

(C) In at least one appropriate chamber whenever a malfunction is detected in any part of the test substance delivery system. Among replicate test chambers of a treatment concentration, the measured concentration of the test substance should not vary more than 20 percent.

(5) [Reserved]

(6) *Analytical measurements*—(i) *Test chemical.* Deionized water should be used in making stock solutions of the test substance. Standard analytical methods should be used whenever available in performing the analyses. The analytical method used to measure the amount of test substance in a sample should be validated before beginning the test by appropriate laboratory practices. An analytical method is not acceptable if likely degradation products of the test substance, such as hydrolysis and oxidation products, give positive or negative interferences which cannot be systematically identified and corrected mathematically.

(ii) *Numerical.* The number of dead shrimp should be counted during each definitive test. Appropriate statistical analyses should provide a goodness-of-fit determination for the concentration-response curves. A 48- and 96-hour $LC_{50}$ and corresponding 95 percent intervals should be calculated.

(d) *Test conditions*—(1) *Test species*—(i) *Selection.* This test should be conducted using one of three species of penaeid shrimp: *Penaeus aztecus* (brown shrimp), *Penaeus duorarum* (pink shrimp), or *Penaeus setiferus* (white shrimp). Post-larval juvenile shrimp should be utilized. Shrimp may be reared from eggs in the laboratory or obtained directly as juveniles or adults. Shrimp used in a particular test should be of similar age and be of normal size and appearance. Shrimp should not be used for a test if they exhibit abnormal behavior or if they have been used in a previous test, either in a treatment or in a control group.

(ii) *Acclimation.* During acclimation, shrimp should be maintained in facilities with background colors and light intensities similar to those of the testing areas. In addition, any change in the temperature and chemistry of the dilution water used for holding and acclimating the test organisms to those of the test should be gradual. Within a 24-hour period, changes in water temperature should not exceed 1 °C, while salinity changes should not exceed 2 percent.

(iii) *Care and handling.* Upon arrival at the test facility, the shrimp should be transferred to water closely matching the temperature and salinity of the transporting medium. Shrimp should be held in glass tanks of 30 liter capacity or larger. No more than 22 to 24 shrimp should be placed in a 30 liter tank unless the flow-through apparatus can maintain dissolved oxygen levels above 60 percent of saturation. With species of the genus *Penaeus*, a minimum flow rate of 7.5 1/g body weight day should be provided. Larger flows, up to 22 1/g body weight day, may be desirable to ensure dissolve oxygen concentrations above 60 percent of saturation and the removal of metabolic products. The period of acclimation to ambient laboratory conditions should be at least 4 to 7 days.

(iv) *Feeding.* Penaeid shrimp should not be fed during testing. Every 2 or 3 days during the acclimation period, shrimp should be fed fish pieces approximately 1 $cm^2$. Uneaten food should be removed daily.

(2) *Facilities*—(i) *Apparatus.* (A) Facilities which may be needed to perform this test include: flow-through tanks for holding and acclimating penaeid shrimp; a mechanism for controlling and maintaining the water temperature and salinity during the holding period; apparatus for straining particulate matter, removing air bubbles, or aerating water when necessitated by water quality requirements; and an apparatus providing a 12-hour light and 12-hour dark photoperiod with a 15 to 30 minute transition period. Facilities should be well ventilated, free of fumes and free of all

other disturbances that may affect test organisms.

(B) Two to three centimeters of acid-washed sand, free of excess organic matter, should be placed in the bottom of test chambers.

(C) Test chambers should be loosely covered to reduce the loss of test solution or dilution water due to evaporation, minimize entry of dust and other particles and prevent escape of the shrimp.

(ii) *Cleaning.* Test substance delivery systems and test chambers should be cleaned before each test following standard laboratory practices.

(iii) *Construction materials.* Materials and equipment that contact test solutions should be chosen to minimize sorption of test chemicals from dilution water and should not contain substances that can be leached into aqueous solution in quantities that can affect test results.

(iv) *Dilution water.* (A) Natural or artificial seawater is acceptable as dilution water if shrimp will survive in it without signs of stress, such as unusual behavior or discoloration. Shrimp should be acclimated and tested in dilution water from the same origin.

(B) Natural seawater should be filtered through a 5 micrometer filter with a pore size $<20$ microns prior to use in a test.

(C) Artificial seawater can be prepared by adding commercially available formulations or by adding specific amounts of reagent-grade chemicals to deionized water. Deionized water with a conductivity less than $1\mu$ ohm/cm at 12 °C is acceptable for making artificial seawater. When deionized water is prepared from a ground or surface water source, conductivity and total organic carbon (or chemical oxygen demand) should be measured on each batch.

(v) *Test substance delivery system.* Proportional diluters, metering pumps or other suitable systems should be used to deliver test substance to the test chambers. The system used should be calibrated before each test. Calibration includes determining the flow rate through each chamber and the concentration of the test substance in each chamber. The general operation of the test substance delivery system should be checked twice daily during a test. The 24-hour flow through a test chamber should be equal to a least 5 times the volume of the test chamber. During a test, the flow rates should not vary more than 10 percent among test chambers or across time.

(3) *Test parameters.* Environmental parameters of the water contained in test chambers should be as specified below:

(i) Temperature of 23 ± 1 °C.

(ii) Dissolved oxygen concentration between 60 and 105 percent saturation. Aeration, if needed to achieve this level, should be done before the addition of the test substance. All treatment and control chambers should be given the same aeration treatment.

(iii) The number of shrimp placed in a test solution should not be so great as to affect results of the test. Loading requirements will vary depending on the flow rate of dilution water. The loading should not cause the dissolved oxygen concentration to fall below the recommended levels.

(iv) Photoperiod of 12 hours light and 12 hours darkness, with a 15 to 30 minute transition period.

(v) Salinity of 20 parts per thousand ±3 percent.

(e) *Reporting.* The sponsor should submit to the EPA all data developed by the test that are suggestive or predictive of acute toxicity and all other toxicological manifestations. In addition to the general reporting requirements prescribed in Part 792—*Good Laboratory Practice Standards* of this chapter, the reporting of test data should include the following:

(1) The nature of the test, laboratory, name of the investigator, test substance and dates of test should be supplied.

(2) A detailed description of the test substances should be provided. This information should include the source, lot number, composition, physical and chemical properties and any carrier or additives used.

(3) Detailed information about the shrimp should be provided: common and scientific names, source of supply, age, history, weight, acclimation pro-

cedure and feeding history should be reported.

(4) A description of the experimental design including the number of test solution concentrations, number of replicates and number of shrimp per replicate should be provided.

(5) The source of the dilution water, its chemical characteristics (e.g., salinity) and a description of any pretreatment.

(6) A description of the test chambers, the depth and volume of solution in the chamber, the number of organisms per treatment, the number of replicates, the loading, the lighting, the test substance delivery system and flow rate expressed as volume additions per 24 hours.

(7) The concentration of the test substance in each test chamber before the start of the test and at the end.

(8) The number of dead shrimp and measurements of water temperature, salinity, and dissolved oxygen concentration in each test chamber should be recorded at the designated times.

(9) Methods and data records of all chemical analyses of water quality and test substance concentrations, including method validations and reagent blanks.

(10) Recorded data for the holding and acclimation period (temperature, salinity, etc.).

(11) Concentration-response curves should be fitted to mortality data collected at 24, 48, 72, and 96 hours. A statistical test of goodness-of-fit should be performed.

(12) For each set of mortality data, the 48- and 96-hour $LC_{50}$ and 95 percent confidence limits should be calculated on the basis of the average measured concentration of the test substance. When data permits, $LC_{50}$ values with 95 percent confidence limits should be computed for 24- and 72-hour observations.

(13) The methods used in calculating the concentration-response curves and the $LC_{50}$ values should be fully described.

## Subpart C—Terrestrial Guidelines

### § 797.2050 Avian dietary toxicity test.

(a) *Purpose.* The guideline in this subpart is designed to develop data on the dietary toxicity to bobwhite and mallard of chemical substances and mixtures subject to acute environmental effects test regulations under the Toxic Substances Control Act (TSCA) (Pub. L. 94-469, 90 Stat. 2003 15 U.S.C. 2601 *et seq.*). The Agency will use these and other data to assess the acute hazard to birds and to provide an indication of potential chronic hazard that these chemicals may present to the environment.

(b) *Definitions.* The definitions in section 3 of the Toxic Substances Control Act (TSCA) and Part 792—*Good Laboratory Practice Standards* of this chapter apply to this test guideline. In addition, the following definitions apply to this guideline:

(1) "Acclimation" Physiological or behavioral adaptation of test animals to environmental conditions and basal diet associated with the test procedure.

(2) "$LC_{50}$" The empirically derived concentration of the test substance in the diet that is expected to result in mortality of 50 percent of a population of birds which is exposed exclusively to the treated diet under the conditions of the test.

(3) "Test Substance" The specific form of a chemical or mixture of chemicals that is used to develop the data.

(4) "Exposure period" The 5-day period during which test birds are offered a diet containing the test substance.

(5) "Post-exposure period" The portion of the test that begins with the test birds being returned from a treated diet to the basal diet. This period is typically 3 days in duration, but may be extended if birds continue to die or demonstrate other toxic effects.

(6) "Test period" The combination of the exposure period and the post-exposure period; or, the entire duration of the test.

(7) "Hatch" Eggs or young birds that are the same age and that are derived from the same adult breeding population, where the adults are of the same strain and stock.

(8) "Basal diet" The food or diet as it is prepared or received from the supplier, without the addition of any carrier, diluent, or test substance.

(c) *Test procedures*—(1) *Summary of test*. (i) After birds have been obtained, they should be acclimated for at least 7 days.

(ii) Test birds should be randomly assigned to the various treatment levels and controls.

(iii) Definitive test concentrations should be established, possibly requiring a range-finding test to be conducted first.

(iv) The test substance should be mixed thoroughly and evenly into the diet. Three treatment levels should be analyzed for test substance concentrations.

(v) Birds should be weighed at the beginning of the exposure period.

(vi) Birds should be observed regularly for mortality or abnormal behavior; any findings should be reported.

(vii) Food treated with the test substance should be replaced by untreated food (basal diet) after 5 days of exposure. Food consumption during the exposure period should be carefully estimated on a pen by pen basis.

(viii) Food consumption should be estimated for the post-exposure period and birds should be weighed at the end of 8 days. Additional weights and food consumption estimates should be determined if the test period is longer than the typical 8 days.

(ix) The mortality pattern should be examined, and a statistical analysis should be conducted. The $LC_{50}$ slope, and confidence limits should be reported. A test for heterogeneity of data should be conducted.

(x) Treated or positive control birds should be sacrificed and disposed of properly. Negative control birds may be kept as breeding stock, but should not be used in any other tests.

(xi) The material to be tested should be analytically pure and the degree of purity should be reported along with the percentage of each impurity at levels specified in the test rule. If specifically required by a test rule for a particular substance or mixture, the technical grade should be tested. The test rule will specify the degree of purity or a range of compositions of the test substance.

(xii) A test is unacceptable if more than 10 percent of the control birds die during the test.

(2) [Reserved]

(3) *Range-finding test*. Unless the approximate toxicity of the test substance is known already, a range-finding test should be conducted to determine the test substance concentrations to be used in the definitive test, under paragraph (c)(4)(iii) of this section for details on concentrations for definitive tests. Procedures for range-finding tests may vary, but generally, groups of a few birds are fed three to five widely-spaced concentrations for 5 days. A concentration series of 5, 50, 500, and 5,000 ppm is suggested. The results of the range-finding test then may be used to establish the definitive test concentrations.

(4) *Definitive test*—(i) *Controls*. (A) A concurrent control is required during every test. The control birds should be from the same hatch as the test groups. Control and test birds should be kept under the same experimental conditions. The test procedures should be the same for control and treated birds, except that no test substance should be added to the diets of control birds. If a carrier is used in preparation of the test diets, the same carrier should be added to the diets of control birds in the highest concentration used for test diets. The use of shared controls is acceptable for concurrent tests as long as the same carrier is used for all the tests.

(B) A test is not acceptable if more than 10 percent of the control birds die during the test period.

(C) A positive control (e.g., dieldrin standard) may be run, but is not required for each test. However, a quarterly or semi-annual laboratory standard (positive control) is recommended as a means of detecting possible interlaboratory or temporal variation. A laboratory standard is also recommended when there is any significant change in food, housing, or source of birds.

(ii) *Number of animals tested*. In the definitive test, a minimum of 10 birds should be used for each dietary concentration of the test substance. A minimum of 20 birds should be used for the negative or carrier control. Thirty or more control birds are preferable. If a positive control or laboratory standard is used, 10 or more birds should also be used for each concentration of the positive control. When a test substance is known or expected to result in high experimental variation, it may be appropriate or required by the test rule to use additional birds.

(iii) *Concentrations and dosage-mortality data.* A minimum of five concentrations of the test substance should be used in the definitive test. These concentrations should be spaced geometrically. The recommended spacing is for each concentration to be at least 60 percent of the next higher dose (less than 1.67 times the next lower dose). If concentrations are spaced more widely than is recommended, then at least three concentrations should result in mortality between, but not including, 0 percent and 100 percent. For any concentration spacing, at least one concentration should kill more than 50 percent (including 100 percent) and at least one concentration should kill less than 50 percent (including 0 percent) of the birds in a pen. For some test substances, it may be necessary to use more than five concentrations to achieve these results.

(iv) *Duration of test.* The definitive test should include 5 days of exposure to the test substance in the diet (exposure period) followed by at least 3 days of additional observation (post-exposure period) while the test birds are receiving an untreated diet. If any test birds die during the second or third day of the post-exposure period or if toxic signs are evident on the third day of the post-exposure period, the test period should be extended until 2 successive mortality-free days and 1 day free of toxic signs occur, or until 21 days after beginning the test, whichever comes first.

(v) *Observations of record.* (A) Throughout the test period, all signs of intoxication, other abnormal behavior, and mortality should be recorded and reported by dose level and by day. Signs of intoxication are those behaviors apparently due to the test chemical and may include a wide array of behaviors, such as labored respiration, leg weakness, hemorrhage, convulsions, ruffled feathers, etc. All signs of intoxication and any other abnormal behavior, such as excessive aggression, toe-picking etc. that may or may not be attributed to the test substance should be reported. Among survivors, remission of signs of intoxication and cessation of abnormal behavior should be recorded by dose level and by day. When differential signs of intoxication are observed within a dose level, an estimate of the number of birds exhibiting such signs should be recorded. Observation of test birds should be made, at a minimum, three times on the first day of the exposure period. Observations also should be made at least daily throughout the remainder of the test period; twice daily observations are recommended, where feasible.

(B) Average body weights of birds should be recorded and reported for each pen within each treatment and control group at the beginning of the exposure period and the end of the normal 3-day post-exposure period of each test. Body weights 72 hours before the exposure period are not required, but would provide valuable base-line data. Average food consumption should be measured in control pens and pens with the second lowest and second highest concentration levels either daily or every other day. Any significant amount of food spilled onto litter pans should be estimated and reported. For all other pens, average food consumption should be measured for both the exposure period and the normal 3-day post-exposure period. If the study is continued beyond 8 days, body weight and food consumption data should be recorded weekly.

(C) Gross pathology examinations are not required, but they may provide valuable information on target site, mode of action, etc.

(5) [Reserved]

(6) *Analytical measurements*—(i) *Statistical analysis.* (A) A statistical analysis should be conducted by transforming the dietary concentrations to logarithmic values and the mortality pattern to probits. Other acceptable methods that will result in a theoretically straight line through ±2 standard deviations from the $LC_{50}$ value may be used. The $LC_{50}$ value and slope of the transformed concentration-response curve should be determined for mortality at the end of test period.

Probit analysis by calculations or graphical probit methods are preferred. Any standard method that is used should provide the slope of the transformed concentration-response curve as well as the $LC_{50}$ value. A statistical test for goodness-of-fit (e.g., chi-square test) also should be performed.

(B) All methods used for statistical analysis should be described completely.

(ii) *Analysis for test substance concentrations.* (A) Samples of treated diets should be analyzed to confirm proper dietary concentration of the test substance. Analyses should be conducted at the beginning of the exposure period with samples from high, middle, and low concentrations. If not already available, data should be generated to indicate whether or not the test substance degrades or volatilizes. If the test substances is known or found to be volatile or labile to the extent that 25 percent or more loss occurs over a 5 day period, then a second series of analyses of the same concentrations previously analyzed should be conducted at the end of the exposure period.

(B) The assay method used to determine actual concentrations should be reported.

(iii) *Analysis of basal diet.* A nutrient analysis of the basal diet should be included in the test report. For commercially prepared basal diets, the list of ingredients supplied by the company is normally sufficient if it is detailed. The composition of any vitamin or other supplements should also be reported.

(d) *Test conditions*—(1) *Test Species*—(i) *Selection.* (A) Bobwhite, *Colinus virginianus* (L.), and mallard, *Anas platyrhynchos* L., are the test species. Birds may be reared in the laboratory or purchased from a breeder. If bobwhite are purchased, it is preferable that they be obtained as eggs which then are hatched and reared in the testing facility. During incubation, a temperature of 39 °C and relative humidity of 70 percent are recommended for bobwhite. It is feasible to purchase live young bobwhite chicks if they can be obtained locally; however, young bobwhite may suffer adverse effects if shipped by air or other commercial means. Young mallard ducklings normally can be shipped without undue adverse effects.

(B) All control and treatment birds used in a test should be from the same source and hatch. Birds should be obtained only from sources whose colonies have known breeding histories. Birds should be phenotypically indistinguishable (except for size) from wild stock. It is recommended that birds be obtained from flocks that have been outbred periodically with genetically wild stock in order to maintain a genetic composition that approximates the natural heterogeneity of the species.

(C) Birds used in the test should be in apparent good health. Deformed, abnormal, sick, or injured birds should not be used. Birds should not be used for a test if more than 5 percent of the total test population die during the 72 hours immediately preceding the exposure period. Purchased birds should be certified as disease free or as bred from disease free stocks. Birds should not have been selected in any way for genetic resistance to toxic substances. Birds should not have been used in a previous test, either in a treatment or control group.

(D) Test birds should be 10 to 17 days old at the beginning of the exposure period. All treatment and control birds in a test should be the same age ± one day. The exact age should be recorded and reported.

(E) Test birds should be acclimated to test facilities and basal diet for a minimum of 7 days. Acclimation to test pens may be either in the actual pens used in the test or in identical pens. Birds used in the test should be assigned randomly to treatment and control pens without respect to sex. Randomization may be done either at the initiation of the acclimation period or at the time when the birds are weighed at the beginning of the exposure period.

(F) During holding, acclimation, and testing, birds should be shielded from excessive noise, activity, or other disturbance. Birds should be handled only as much as is necessary to conform to test procedures.

(ii) *Diet.* (A) A standard commercial game bird (for bobwhite) or duck (for mallard) starter mash, or the nutritional equivalent, should be used for diet preparation. Antibiotics or other medication should not be used in the diet before or during the test. For bobwhite only, an antibiotic demonstrated to fully depurate in 72 hours may be added to the drinking water, if necessary, for birds up through 10 days of age; however, only clean unmedicated water should be offered during the 96 hours preceding the exposure period and during the test period. It may not be possible to obtain food that is completely free of pesticides, heavy metals, and other contaminants; however, diets should be analyzed periodically, and should be selected to be as free from contaminants as possible. A nutrient analysis and list of the ingredients in the diet should be included with the test report.

(B) The test substance should be mixed into the diet in a manner that will result in even distribution of the test substance throughout the diet. If possible, the test substance should be added to the diet without the use of a diluent. If a diluent is needed, the preferred diluent is distilled water; but water should not be used as a diluent for test substances known to hydrolyze readily. When a test substance is not water soluble, it may be dissolved in a reagent grade evaporative diluent (e.g., acetone, methylene chloride) and then mixed with the test diet. The diluent should be completely evaporated prior to feeding. Other acceptable diluents may be used, if necessary, and include table grade corn oil, propylene glycol, and gum arabic (acacia). If a diluent is used, it should not comprise more than 2 percent by weight of the treated diet, and an equivalent amount of diluent should be added to control diets for untreated birds.

(C) Diets can be mixed by commercial, mechanical food mixers. For many test substances, it is recommended that treated diets be mixed under a hood. Mashes and test substances should be mixed freshly just prior to the beginning of the test. For certain volatile or other test substances, the Test Rule may require preparation of fresh diets at frequent intervals. Analysis of the diet for test substance concentrations is required under paragraph (c)(6)(ii) of this section.

(D) Clean water should be available *ad libitum.* Water bottles or automatic watering devices are recommended. If water pans or bowls are used, water should be changed at least once a day.

(2) *Facilities.* (i) Tests should be conducted with birds being maintained in commercial brooder pens or pens of similar construction. Pens should be constructed of galvanized metal, stainless steel, or perfluorocarbon plastics. Materials that are toxic, may affect toxicity, or may adsorb test substances should not be used. Wire mesh should be used for floors and external walls; solid sheeting should be used for common walls and ceilings. Wire mesh for floors should be fine enough so as to not interfere with the normal movement of young birds. Pens for housing 10 young birds should have a floor area of at least 3,000 square centimeters (approximately 500 square inches) for bobwhite and 6,000 square centimeters (approximately 1,000 square inches) for mallards and should be at least 24 centimeters (approximately 9.5 inches) high. Pens should be disassembled (if feasible) and should be cleaned thoroughly between tests. Steam cleaning of cages is recommended. Cages may be brushed thoroughly, as an alternative method. The use of detergents or bleach is acceptable, but other chemical disinfectants such as quaternary ammonium compounds should not be used. When necessary to control disease vectors, hot or cold sterilization techniques are recommended, as long as such techniques will not leave chemical residues on the cages. For cold sterilization, ethylene oxide is recommended. Pens should not be cleaned during a test.

(ii) Pens should be kept indoors to control lighting, temperature, and other environmental variables. Pens should be heated, preferably by thermostatic control. A temperature gradient in the pen of approximately 35 °C to approximately 22 °C will allow young birds to seek a proper temperature. Temperature requirements for young birds typically decline over this range from birth through the first several weeks of life. Relative humidity is not as critical, but the test room should be maintained at a relative humidity of 45-70 percent. A photoperiod of 14 hours light and 10 hours dark is recommended. Other light/dark cycles should not be used, but continuous lighting is acceptable. Lighting may be either incandescent or fluorescent. Pens and lights should be positioned so that all pens will receive similar illumination. The facilities should be well ventilated.

(iii) Where feasible, it is recommended that pens not be stacked upon each other. If pens are stacked, only one test substance is allowed in any single stack. If a test substance volatilizes or otherwise forms aerosols or vapors in the air, no more than one test substance should be tested in a room in order to avoid cross-contamination. Pens should be randomly arranged, whether or not in a stack, with respect to dose levels and controls. Pens, such as stacked, unmodified, commercial pens with external feeders, that allow food to be spilled from one pen to a lower pen, should be avoided. Any modifications that prevent cross contamination of concentration levels are acceptable. For example, commercially available, 30 cm (one foot) long chick feeders may be placed inside the pens and be covered with 1.27 cm (0.5 inch) mesh hardware cloth over the food, for bobwhite. The same feeders covered with approximately 2.5 cm (one inch) mesh wire are appropriate for mallards. For either species, external feeders can be covered with the appropriate size wire mesh and a solid piece of metal extended from the bottom of the cage to a point exterior to the feeder. Spillage may occur, but the added metal will prevent food from spilling into another feeder.

(e) *Reporting.* (1) The test report should include the following information:

(i) Name of test, sponsor, test laboratory and location, principal investigator(s), and actual dates of beginning and end, of test.

(ii) Name of species tested (including scientific name), age of birds (in days) at the beginning of the test, average body weights for birds in each pen at the beginning of the test, the end of the exposure period, and end of the test, and individual weights of all birds that die during the test.

(iii) Description of housing conditions, including type, size, and material of pen, pen temperatures, approximate test room humidity, photoperiod and lighting intensity.

(iv) Detailed description of the basal diet, including source, diluents (if used), and supplements (if used). A nutrient analysis of the diet should be included in the test report.

(v) Detailed description of the test substance including its chemical name(s), source, lot number, composition (identity of major ingredients and impurities), and known physical and chemical properties that are pertinent to the test (e.g., physical state, solubility, etc.).

(vi) The number of concentrations used, nominal and (where required) measured dietary concentration of test substance in each level, assay method used to determine actual concentrations, number of birds per concentration and for controls, and names of toxicants used for positive controls (if applicable).

(vii) Acclimation procedures and methods of assigning birds to test pens.

(viii) Frequency, duration, and methods of observation.

(ix) Description of signs of intoxication and other abnormal behavior, including time of onset, duration, severity (including death), and numbers affected in the different dietary concentrations and controls each day of the test period.

(x) Estimated food consumption per pen daily or every other day in the second highest and second lowest concentration and control pens. For other pens, food consumption should be estimated for the exposure period and for the post-exposure period.

(xi) Location of raw data storage.

(xii) Results of range finding tests (if conducted).

(xiii) The calculated $EC_{50}$ value, 95 percent confidence limits, slope of the concentration-response curve, the results of the goodness-of-fit test (e.g., chi-square test), and a description of statistical methods used. The same statistics for positive controls (when used). The methods used for statistical analysis should be described completely.

(xiv) Anything unusual about the test, any deviation from these procedures, and any other relevant information.

(2) In addition to the above information required in every report, the following information should be available upon request:

(i) A general description of the support facilities.

(ii) A description of the Quality Control/Quality Assurance program, including the Average Quality Level for the program element performing the test, procedures used, and documentations that these levels have been achieved.

(iii) The names, qualifications, and experience of personnel working in the program element performing the test, including the study director, principal investigator, quality assurance officer, as well as other personnel involved in the study.

(iv) Standard operating procedures for all phases of the test and equipment involved in the test.

(v) Sources of all supplies and equipment involved in the test.

(vi) Originals or exact copies of all raw data generated in performing the test.

[50 FR 39321, Sept. 27, 1985, as amended at 53 FR 12526, Apr. 15, 1988]

## § 797.2130 Bobwhite reproduction test.

(a) *Purpose.* This guideline is designed to develop data on the reproductive effects on the bobwhite of chemical substances and mixtures subject to chronic environmental effects test regulations under the Toxic Substances Control Act (TSCA) (Pub. L. 94-469, 90 Stat. 2003 15 U.S.C. 2601 *et seq.*). The Agency will use these and other data to assess the reproductive effects on birds that these chemicals may present to the environment.

(b) *Definitions.* (1) The definitions in section 3 of the Toxic Substances Control Act (TSCA) and Part 792—*Good Laboratory Practice Standards* of this chapter apply to this test guideline. In addition, the following definitions apply generally to this guideline:

(i) "Acclimation" Physiological and behavioral adaptation to environmental conditions (e.g., housing and diet) associated with the test procedure.

(ii) "Test substance" The specific form of a chemical or mixture of chemicals that is used to develop the data.

(iii) "Photoperiod" The light and dark periods in a 24 hour day. This is usually expressed in a form such as 17 hours light/7 hours dark or 17L/7D.

(iv) "Basal diet" The untreated form of the diet, such as the diet obtained from a commercial source.

(2) The definitions in this section refer specifically to the production and quality of eggs and the subsequent development of these eggs up to the point where young are 14 days old.

(i) "Eggs laid" This term refers to the total egg production during the test, which normally includes 10 weeks of laying. Values are expressed as numbers of eggs per pen per season (or test).

(ii) "Eggs cracked" Eggs determined to have cracked shells when inspected with a candling lamp. Fine cracks cannot be detected without using a candling lamp and if undetected will bias data by adversely affecting embryo development. Values are expressed as a percentage of eggs laid by all hens during the test.

(iii) "Eggs set" All eggs placed under incubation, i.e., total eggs minus cracked eggs and those selected for analysis of eggshell thickness. The number of eggs set, itself, is an artificial number, but it is essential for the statistical analysis of other development parameters.

(iv) "Viable embryos (fertility)" Eggs in which fertilization has occurred and embryonic development has begun. This is determined by candling the eggs 11 days after incubation has begun. It is difficult to distinguish between the absence of fertilization and early embryonic death. The distinction can be made by breaking out eggs that appear infertile and examining further. This distinction is especially important when a test compound induces early embryo mortality. Values are expressed as a percentage of eggs set.

(v) "Live 18-day embryos" Embryos that are developing normally after 18 days of incubation. This is determined by candling the eggs. Values are expressed as a percentage of viable embryos (fertile eggs).

(vi) "Hatchability" Embryos that mature, pip the shell, and liberate themselves from the eggs on day 23 or 24 of incubation. Values are expressed as percentage of viable embryos (fertile eggs).

(vii) "14-day old survivors" Birds that survive for 2 weeks following hatch. Values are expressed both as a percentage of hatched eggs and as the number per pen per season (test).

(viii) "Eggshell thickness" The thickness of the shell and the membrane of the egg at several points around the girth after the egg has been opened, washed out, and the shell and membrane dried for at least 48 hours at room temperature. Values are expressed as the average thickness of the several measured points in millimeters.

(c) *Test procedures*—(1) *Summary of the test.* (i) After birds have been obtained they should be observed for health and acclimated for at least 2 weeks.

(ii) Test birds should be randomly assigned to control and various treatment groups.

(iii) The test substance should be thoroughly and evenly mixed into the diet at concentrations specified in the test rule. All treatment levels should be analyzed for test substance concentrations at the beginning and midway through the test.

(iv) Birds should be weighed at the beginning of the test, at 14-day intervals until the onset of laying, and at termination of the test.

(v) Photoperiod should be carefully controlled on a shortday basis during the initial exposure phase, then increased to 16 to17 hours to induce egg laying.

(vi) Birds should be observed regularly for abnormal behavior or mortality throughout the test.

(vii) Eggs should be removed daily and stored until there is a sufficient quantity for incubation. All eggs should be candled for cracks and cracked eggs removed. Once every 2 weeks, all eggs produced that day should be analyzed for eggshell thickness. Incubated eggs should be candled on day 11 and day 18. Hatching should be completed by day 24.

(viii) Hatchlings should be maintained until they are 14 days old. Abnormal behavior or death should be reported. Chicks should be weighed on day 14.

(ix) A statistical analysis should be performed, preferably by analysis of variance or regression analysis.

(x) The report should include all conditions, procedures, and results. Data should be sufficiently detailed for an independent statistical analysis.

(xi) All treated birds should be sacrificed and disposed of properly. Control birds may be kept as breeding stock, but should not be used in any other tests. Control offspring may be reared and used in another test as adults.

(2)—(3) [Reserved]

(4) *Definitive test*—(i) *Test substance.* (A) The concentrations of test substance in the diet will be specified in the test rule. Generally, three treatment groups and a control group will be used. The higher two treatment concentrations will be multiples (often 5×, 10×, or 20×) of the lowest treatment level. The highest treatment levels usually will be below lethal levels, unless predicted environmental exposure levels are high enough to approximate lethal concentrations.

(B) The material to be tested should be analytically pure and the degree of purity should be reported along with the percentage of each impurity at levels specified in the test rule. If specifically required by a test rule for a particular substance or mixture, the technical grade should be tested. The test rule will specify the degree of purity or a range of compositions of the technical grade material.

(ii) *Controls.* A concurrent control is required during every test. The control birds should be from the same hatch as the test groups. Control and test birds should be kept under the same experimental conditions. The test procedures should be the same for control and treated birds, except that no test substance should be added to the diets of control birds. If a carrier or diluent is used in preparation of the test diets, the same carrier should be added to the diets of control birds in the highest concentration used for test diets. The use of shared controls is acceptable for concurrent tests as long as the same carrier is used for all the tests.

(iii) *Test groups and numbers of birds.* (A) Each of the three treatment groups and the control group should consist of a minimum of 12 replicate pens. Each pen should contain one male and one female, or alternatively one male and two females. The use of 20 replicate pens in the control group may yield a test with greater statistical power. Either arrangement is acceptable if productivity reaches the definitive values given in paragraph (c)(4)(xii) of this section. Because the behavioral interactions of birds in the two arrangements are likely to be different, testing facilities using an arrangement with which they are not familiar are advised to experiment first without test substances in order to determine the feasibility of obtaining acceptable productivity levels.

(B) All control and treatment birds should be randomly distributed to pens from the same population.

(iv) *Duration of test.* (A) The test consists of three phases following acclimation to test facilities. The initial phase begins with exposure of treatment groups to diets containing the test substance and is typically 6 to 8 weeks long. After the initial phase, the photoperiod is manipulated according to paragraph (c)(4)(v) of this section to bring the hens into laying condition. This second phase ends with the onset of egg-laying and is typically 2 to 4 weeks long. The final phase begins with the onset of laying and lasts for at least 8 weeks, preferably 10 weeks. A withdrawal study period may be added to the test phase if reduced reproduction is observed. The withdrawal period, if used, need not exceed 3 weeks.

(B) Exposure of adult birds to the test substance should be continuous throughout the test. Unless otherwise specified in the test rule, test birds should be exposed for at least 10 weeks prior to the onset of egg laying.

(v) *Preparation for reproduction (photoperiod).* (A) Lighting regimes (photoperiod) are critical to successful reproduction. Various photoperiod regimes have been demonstrated to give acceptable results. Any photoperiod regime that results in productivity that meets the definitive values given in paragraph (c)(4)(xii) of this section is acceptable as long as birds are exposed to treated diets a minimum of 10 weeks prior to the onset of laying. Regardless of the methods selected, lighting should be controlled carefully. It is important during the initial phase to not interrupt the dark period unless absolutely necessary.

(B) A suggested photoperiod regime would consist of maintaining birds under a photoperiod for 7 or 8 hours of light during the initial phase. At the end of the initial phase, the photoperiod may be increased to 16 to 17 hours of light per day. The photoperiod may be maintained at this level for the remainder of the study, although an increase each week of 15 minutes per day is acceptable.

(vi) *Observations of record on adult birds.* (A) Body weights should be recorded for each adult bird at the beginning of the treatment period, at 14-day intervals until the onset of egg laying, and at termination of treatment. Recording of body weights during egg laying is discouraged because of possible adverse effects on egg production. Food consumption should be measured and recorded by pen as often as body weights are measured prior to the onset of laying and at least bi-weekly throughout the rest of the study.

(B) Observations on adult birds should be made at least once a day. Any mortality or other signs of toxicity should be described and recorded by date or day of test. Gross pathological examinations should be conducted on all birds that die during the test period, and for all survivors at the end of the test. Analysis of two or more tissues (e.g., muscle, fat) for test substance residues is encouraged, but not required (unless specified in the Test Rule).

(vii) *Egg collection, storage, and incubation.* All eggs should be collected daily, marked according to the pen from which collected, and should be stored at 16 °C and 55 to 80 percent relative humidity. Storage in plastic bags may improve uniformity of hatching. Stored eggs should be turned daily. At weekly or bi-weekly intervals, eggs should be removed from storage and be candled to detect eggshell cracks. Except for eggs with cracked shells and those eggs removed for eggshell thickness measurements, all eggs should be set after candling for incubation in a commercial incubator. If incubators are not equipped to automatically turn eggs, they should be turned daily by hand. During the incubation period, eggs should be maintained at 37.5 °C and approximately 70 percent relative humidity. Eggs should be candled again on day 11 of incubation to determine fertility and early death of embryos. A final candling should be done on day 18 to measure embryo survival. On day 21, eggs should be removed to a separate incubator or hatcher. Hatching will normally be complete by the end of day 24.

(viii) *Chick maintenance.* By day 24 of incubation, the hatched bobwhite chicks should be removed from the hatcher or incubator. Chicks should be either housed according to the appropriate parental pen group or individually marked (such as by leg bands) as to parental group and housed together. Chicks should be maintained in commercial brooder pens or pens of similar construction. Pens should be constructed of galvanized metal or stainless steel. Temperature in the pens should be controlled, preferably by a thermostatically controlled device. A temperature gradient in the pen from approximately 35 °C to approximately 22 °C will allow young birds to seek a proper temperature. Temperature requirements for young birds typically decline over this range from birth through the first several weeks of life. Chicks should be provided a standard commercial game bird starter ration, or its nutritional equivalent. No test substance may be added to the diet of chicks. Chicks should be maintained until they are 14 days old.

(ix) *Observations of record on chicks.* The hatchability, percentage of normal hatchlings, percentage of 14-day old survivors, and number of 14-day old survivors per hen should be recorded and reported. Chicks should be observed daily from hatching until they are 14 days old. Mortality, signs of toxicity, and other clinical abnormalities should be recorded at least cumulatively through day 5 and recorded by age from days 5 through 14. Average body weights should be recorded for chicks at day 14.

(x) *Eggshell thickness.* Once every two weeks all eggs newly laid that day should be removed and measured for eggshell thickness. Eggs should be opened at the girth (the widest portion), the contents washed out (or used or saved for egg residue analysis), and the shell air dried for at least 48 hours. The thickness of the shell plus the dried membrane should be measured at a minimum of 3 points around the girth using a micrometer calibrated at least to 0.01 mm units.

(xi) *Typical observed values.* The values reported here represent those observed from a few testing facilities under their conditions. These values are not necessarily representative of those from all facilities, however, if a reproduction test does not meet or at least approach these values for control birds, then there is likely to be a problem with test procedures or conditions that should be investigated and corrected.

(A) *Eggs laid.* Normal values for bobwhite—28 to 38 eggs per hen per season.

(B) *Eggs cracked.* Normal values for bobwhite—0.6 to 2.0 percent of eggs laid.

(C) *Viable embryos (fertility).* (Normal fertility values for bobwhite—75 to 90 percent of eggs set.

(D) *Live 18-day embryos.* Normal values for bobwhite—97 to 99 percent of viable embryos.

(E) *Hatchability.* Normal values for bobwhite—50 to 90 percent of viable embryos (fertile eggs).

(F) *14-day-old survivors.* Normal values for bobwhite—75 to 90 percent of eggs hatched.

(G) *Eggshell thickness.* Normal average values for bobwhite—0.19 to 0.24 mm.

(xii) *Definitive test criteria.* (A) A test is unacceptable if bobwhite chick productivity in control groups does not average twelve 14-day old survivors per pen over a 10-week period.

(B) A test is unacceptable if the average eggshell thickness in control groups is less than 0.19 mm.

(C) A test is unacceptable if more than 10 percent of the adult control birds die during the test.

(5) [Reserved]

(6) *Analytical measurements*—(i) *Statistical analysis.* Experimental groups should be individually compared to the control group by analysis of variance. Other accepted statistical methods may be used as long as they are documented. In particular, regression analysis is highly desirable if the data and number of dose levels allow the use of this technique. Sample units are the individual pens within each treatment level or control. Analysis should include:

(A) Body weight of adults.

(B) Food consumption of adults.

(C) Percentage of hens laying eggs. This should always be determined when pens contain a single pair; if feasible, it should be determined when pens contain groups.

(D) Number of eggs laid per pen.

(E) Percentage of cracked eggs.

(F) Percent viable embryos of eggs set.

(G) Percent live 18-day embryos of viable embryos.

(H) Percent hatching of viable embryos.

(I) Percentage of hatchlings that are normal.

(J) Percent 14-day-old survivors of normal hatchlings.

(K) Number of 14-day-old survivors per hen.

(L) Body weights of 14-day-old survivors.

(M) Eggshell thickness.

(ii) *Test substance concentrations.* (A) Samples of treated diets should be analyzed to confirm proper dietary concentrations of the test substance. If samples cannot be analyzed immediately, they should be stored appropriately (e.g., frozen at a temperature of −15 °C or lower) until analysis can be performed. Analyses should be conducted on all test substance concentrations at the beginning of the test period and again 10 to 12 weeks later. If not otherwise available, data should be generated to indicate whether or not the test substance degrades or volatilizes. If the test substance is known or found to be volatile or labile to the extent that 25 percent or more loss occurs within 1 week, then test substance diets should be prepared (freshly or from frozen concentrate) at a frequency that will prevent more than 25 percent loss of test substance.

(B) The assay method used to determine actual concentrations should be reported according to paragraph (e)(1)(vi) of this section.

(iii) *Analysis of Basal Diet.* A nutrient analysis of the basal diet should be included with the test report. For commercially prepared basal diets, the list of ingredients supplied by the manufacturer is normally sufficient if it is detailed. The composition of any vitamin or other supplements should also be reported.

(d) *Test conditions*—(1) *Test species*—(i) *Selection.* (A) Bobwhite, *Colinus virginianus* (L.), is the test species. Test birds should be pen-reared. They may be reared in the laboratory or purchased from commercial breeders. Rearing stock and/or tests birds should be obtained only from sources that have met the requirements for "U.S. Pullorum-Typhoid Clean" classification under paragraph (f)(1) of this section. Birds should be obtained only from sources whose colonies have known breeding histories. If possible, a history of rearing practices for test birds should be obtained and made available upon request. This history should include lighting practices during rearing, disease record, drug and any other medication administered, and exact age. Test birds should be phenotypically indistinguishable (except for size) from wild stock. Conscientious breeders of such birds will periodically outbreed their flocks with genetically wild stock in order to maintain a genetic composition that approximates the heterogeneity of naturally occurring birds.

(B) All control and experimental birds used in a test should be from the same source and strain. If shipped, all birds should be examined following shipment for possible physical injury that may have occurred in transit. All birds should have a health observation period of at least 2 weeks prior to selection for treatment. Birds should be in apparent good health. Deformed, abnormal, sick, or injured birds should not be used. A population of birds should not be used if more than 3 percent of either sex die during the health observation period. Birds should not have been selected in any way for resistance to toxic substances. Birds should not have been used in a previous test, either in a control or treatment group. Offspring of birds used in a treatment group in a previous test should not be used, but offspring of birds used as a control in a previous test are acceptable.

(C) Tests birds should be approaching their first breeding season and should be at least 7 months old. All test birds should be the same age within 1 month. The age of test birds should be reported.

(D) Bobwhite should be acclimated to test facilities and untreated basal diet for at least 2 weeks. Acclimation may be in the actual pens used in the test or in identical pens. The acclimation period may coincide with the health observation period. Birds should be randomly assigned to treatment and control pens. However, when birds in a pen are incompatible, they may be rearranged within a control or treatment group at any time prior to initiating treatment.

(E) During holding, acclimation, and testing, birds should be shielded from excessive noise, activity, or other disturbance. Birds should be handled only as much as is necessary to conform to test procedures.

(ii) *Diet*—(A) *Adult birds.* (*1*) A standard commercial game bird breeder ration, or its nutritional equivalent, should be used for diet preparation. This ration or basal diet should be used for both control and treatment birds and should be constant throughout the duration of the study. Antibiotics or other medication should not be used in the diet or water of breeding birds. It may not be possible to obtain food that is completely free of pesticides, heavy metals, and other contaminants. However, diets should be analyzed periodically for these substances and should be selected to be as free from contaminants as possible. A nutrient analysis (quantitative list of ingredients) of the diet should be included with the test report.

(*2*) The test substance should be mixed into the diet in a manner that will ensure even distribution of the test substance throughout the diet. If possible, the test substance should be added to the diet without the use of a carrier or diluent. If a diluent is needed, the preferred diluent is distilled water; but water should not be used for test substances known to hydrolyze readily. When a test substance is not water soluble, it may be dissolved in a reagent grade evaporative diluent (e.g., acetone, methylene chloride) and then mixed with the test diet. The solvent should be completely evaporated prior to feeding. Other acceptable diluents may be used, if necessary, and include table grade corn oil, propylene glycol, and gum arabic (acacia). If a diluent is used, it should comprise no more than 2 percent by weight of the treated diet, and an equivalent amount of diluent should be added to control diets.

(*3*) Diets may be mixed by commercial or mechanical food mixers. Other means are acceptable as long as they result in even distribution of the test substance throughout the diet. Screening of the basal diet before mixing is suggested to remove large particles. For many tests substances, it is recommended that diets be mixed under a hood. Frequently, the test substance is added to an aliquot of the basal diet to form a premix concentrate. The premix concentrate should be stored so as to maintain the chemical concentration. For final preparation of test diets, the premix is mixed with additional basal diet to form the proper concentrations. The frequency with which final treated diets are prepared will depend upon the stability and other characteristics of the test substance. Unless otherwise specified in the test rule or determined by degradation or volatility studies, it is recommended that final diets be prepared weekly, either fresh or from a concentrate. For volatile or labile test substances, test diets should be mixed frequently enough so that the concentrations are not reduced from initial concentrations by more than 25 percent.

Analysis of diets for test substance concentration is required as specified in paragraph (c)(6)(ii) of this section.

(*4*) Clean water should be available *ad libitum*. Water bottles or automatic watering devices are recommended. If water pans or bowls are used, water should be changed daily or more often.

(B) *Young birds.* Young birds produced during the test should be fed a commercial game bird starter ration, or its nutritional equivalent. No test substance should be added to the diets of young birds. No antibiotics or medication may be used in the diet. Bacitracin, or one of its forms, may be added to the drinking water of young birds, if necessary.

(2) *Facilities.* (i) Bobwhite should be housed in breeding pens or cages of adequate size conforming to good husbandry practices. Space requirements for bobwhite have not been well defined, but it is recommended that there be at least 5000 square centimeters (approximately 2.7 square feet) of floor space per bird. Documentation that reproductive parameters and health of birds are not adversely affected should be provided for cages much smaller than this area. The preferred construction materials are stainless steel, galvanized sheeting, and wire mesh. For enclosed cages, floors and external walls may be wire mesh; ceilings and common walls should be solid sheeting. Wire mesh for floors should be fine enough so as to not interfere with normal movement of bobwhite. Open-topped pens may be constructed of the same materials for the side walls with open tops and wire mesh or concrete floors. Concrete floors should be covered with litter such as straw, wood shavings, or sawdust. Other construction materials, except wood, are acceptable if they can be kept clean. Wood may be used as vertical framing posts for the support of wire mesh or for horizontal framing along the top of the pen. Wood should not be used for floors or lower sides of pens unless it has been coated with a non-adsorbent material such as perfluorocarbon plastic (e.g., Teflon), or unless the wood is replaced between tests.

(ii) Pens should be disassembled (if feasible) and should be cleaned thoroughly between tests. Steam cleaning of enclosed cages is recommended. Enclosed cages may be brushed thoroughly, as an alternative method. For open-topped pens, the sides and vertical supports should be thoroughly brushed. Any used floor litter should be discarded. The floor composition will dictate methods used to clean the floor. If litter is used on the floor, it should be fresh and clean when birds are placed in the pen. The use of detergents or bleach is acceptable, but other chemical disinfectants (such as quaternary ammonium compounds) should not be used. When necessary to control disease vectors, hot or cold sterilization techniques are recommended, as long as such techniques will not leave chemical residues on the cages. For cold sterilization, ethylene oxide is recommended.

(iii) Pens should be kept indoors in order to better control lighting, temperature, humidity, and other factors. Outdoor pens may be used only during the normal breeding season. The photoperiod should be carefully controlled, preferably by automatic timers. A 15 to 30 minute transition period is desirable. The photoperiod regime is described under test procedures under paragraph (c)(4)(v) of this section. Lights should emit a spectrum simulating that of daylight. The use of shorter wave-length "cool-white" fluorescent lights that do not emit the daylight spectrum should be avoided. Illumination intensity should be about 6 foot-candles at the level of the birds.

(iv) Temperature and humidity should be controlled during the study. Recommended levels are 21 °C and 55 percent relative humidity. Temperature should be recorded at least weekly at the same time of day and should be reported. For tests conducted without temperature control, temperature minimums and maximums should be recorded daily. Continuous temperature monitoring is desirable. Temperature recordings should be made at a level of 2.5 to 4 cm above the floor of the cage. Recording of approximate humidity levels is also desirable. Good ventilation should be maintained. Suggested ventilation rates are 4 changes per hour in winter and 15 changes per hour in summer.

(v) If facilities are being used for the first time, it may be desirable to allow birds to breed in the facility prior to testing in order to ensure that controls will have acceptable productivity according to the requirements given in paragraphs (c)(4) (xi) and (xii) of this section.

(e) *Reporting.* (1) The test report should include the following information:

(i) Name of test, sponsor, test laboratory and location, principal investigator(s), and actual dates of beginning and end of test.

(ii) Name of species tested (including scientific name), age of birds (in months) at the beginning of the test, source of birds, and body weights for adult birds throughout the test.

(iii) Description of housing conditions, including type, size, and material of pen, temperature, humidity, photoperiod and lighting intensity, and any changes during the test.

(iv) Detailed description of the basal diet, including source, composition, diluents (if used), and supplements (if used). A nutrient analysis of the basal diet should be included.

(v) Detailed description of the test substance including its chemical name(s), source, lot number, composition (identity of major ingredients and impurities), and known physical and chemical properties pertinent to the test (e.g., solubility, volatility, degradation rate, etc.).

(vi) The number of concentrations used, nominal and measured concentrations of test substance in each level, assay method used to determine actual concentrations, storage conditions and stability of treated diets, number of birds per pen and number of replicate pens per concentration and for controls.

(vii) Acclimation procedures and methods of assigning birds to test pens, including method of randomization, and any rearrangement due to incompatibility.

(viii) Frequency, duration, and methods of observation.

(ix) Description of any signs of intoxication, including time of onset, duration, severity (including death), and numbers affected, including accidental deaths or injuries.

(x) Food consumption per pen and any observations of repellancy or food palatability.

(xi) Method of marking all birds and eggs.

(xii) Details of autopsies.

(xiii) Egg and hatching data in summary and by pen per week in sufficient detail to allow an independent statistical analysis. Data should be presented for all of the parameters listed in paragraph (c)(6)(i) of this section. The number of eggs set should also be reported.

(xiv) Egg storage, incubation, and hatching temperatures, relative humidities, and turning frequencies.

(xv) Observations of health and weights of young at 14 days of age.

(xvi) Location of all raw data storage.

(xvii) Methods of statistical analysis and interpretation of results.

(xviii) Anything unusual about the test, any deviation from these procedures, and any other relevant information.

(2) In addition, the following information should be available upon request:

(i) A general description of the support facilities.

(ii) A description of the Quality Control/Quality Assurance program, including the Average Quality Level for the program element performing the test, procedures used, and documentations that these levels have been achieved.

(iii) The names, qualifications, and experience of personnel working in the program element performing the test, including the study director, principal investigator, quality assurance officer, as well as other personnel involved in the study.

(iv) Standard operating procedures for all phases of the test and equipment involved in the test.

(v) Sources of all supplies and equipment involved in the test.

(vi) Diagram of the test layout.

(vii) Originals or exact copies of all raw data generated in performing the test.

(viii) A detailed description, with references, of all statistical methods.

(f) *References.* For additional background information on this test guideline the following references should be consulted:

(1) U.S. Department of Agriculture. "National Poultry Improvement Plan," Report No. 2. *Directory of Participants Handling Waterfowl, Exhibition Poultry, and Game Birds.* U.S.D.A., Science and Education Administration, Beltsville, MD 20705 (1979).

(2) [Reserved]

### § 797.2150 Mallard reproduction test.

(a) *Purpose.* This guideline is designed to develop data on the reproductive effects on the mallard of chemical substances and mixtures subject to chronic environmental effects test regulations under the Toxic Substances Control Act (TSCA) (Pub. L. 94-469, 90 Stat. 2003 15 U.S.C. 2601 *et seq.*). The Agency will use these and other data to assess the reproductive effects on birds that these chemicals may present in the environment.

(b) *Definitions.* (1) The definitions in section 3 of the Toxic Substances Control Act (TSCA) and Part 792—*Good Laboratory Practice Standards* of this chapter apply to this test guideline. In addition, the following definitions apply generally to this guideline:

(i) "Acclimation" Physiological and behavioral adaptation to environmental conditions (e.g., housing and diet) associated with the test procedure.

(ii) "Test substance" The specific form of a chemical or mixture of chemicals that is used to develop the data.

(iii) "Photoperiod" The light and dark periods in a 24 hour day. This is usually expressed in a form such as 17 hours light/7 hours dark or 17L/7D.

(iv) "Basal diet" The untreated form of the diet, such as the diet obtained from a commercial source.

(2) The definitions in this section refer specifically to the production and quality of eggs and the subsequent development of these eggs up to the point where young are 14 days old.

(i) "Eggs laid" this term refers to the total egg production during the test, which normally includes 10 weeks of laying. Values are expressed as numbers of eggs per pen per season (or test).

(ii) "Eggs cracked" Eggs determined to have cracked shells when inspected with a candling lamp. Fine cracks cannot be detected without using a candling lamp and if undetected will bias data by adversely affecting embryo development. Values are expressed as a percentage of eggs laid by all hens during the test.

(iii) "Eggs set" All eggs placed under incubation, i.e., total eggs minus cracked eggs and those selected for analysis of eggshell thickness. The number of eggs set, itself, is an artificial number, but it is essential for the statistical analysis of other development parameters.

(iv) "Viable embryos (fertility)" Eggs in which fertilization has occurred and embryonic development has begun. This is determined by candling the eggs 14 days after incubation has begun. It is difficult to distinguish between the absence of fertilization and early embryonic death. The distinction can be made by breaking out eggs that appear infertile and examining further. This distinction is especially important when a test compound induces early embryo mortality. Values are expressed as a percentage of eggs set.

(v) "Live 21-day embryos" Embryos that are developing normally after 21 days of incubation. This is determined by candling the eggs. Values are expressed as a percentage of viable embryos (fertile eggs).

(vi) "Hatchability" Embryos that mature, pip the shell, and liberate themselves from their eggs on day 25, 26, or 27 of incubation. Values are expressed as a percentage of viable embryos (fertile eggs).

(vii) "14-day old survivors" Birds that survive for 2 weeks following hatch. Values are expressed both as a percentage of hatched eggs and as the number per pen per season (test).

(viii) "Eggshell thickness" The thickness of the shell and the membrane of the egg at several points around the girth after the egg has been opened, washed out, and the shell and membrane dried for at least 48 hours at room temperature. Values are expressed as the average thickness of the several measured points in millimeters.

(c) *Test Procedures*—(1) *Summary of test.* (i) After birds have been obtained they are observed for health and acclimated for at least 2 weeks.

(ii) Test birds are randomly assigned to control and various treatment groups.

(iii) The test substance is thoroughly and evenly mixed into the diet at concentrations specified in the test rule. All treatment levels are analyzed for test substance concentrations at the beginning and midway through the test.

(iv) Birds are weighed at the beginning of the test, at 14-day intervals until the onset of laying, and at termination of the test.

(v) Photoperiod is carefully controlled on a shortday basis during the initial exposure phase, then increased to 16 to 17 hours to induce egg laying.

(vi) Birds are observed regularly for abnormal behavior or mortality throughout the test.

(vii) Eggs are removed daily and stored until there is a sufficient quantity for incubation. All eggs are candled for cracks, and cracked eggs are removed. Once every 2 weeks, all eggs produced that day are analyzed for eggshell thickness. Incubated eggs are candled on day 14 and day 21. Hatching should be completed by day 27.

(viii) Hatchlings are maintained in pens until they are 14 days old. Abnormal behavior or death is reported. Ducklings are weighed on day 14.

(ix) A statistical analysis is performed, preferably by analysis of variance or regression analysis.

(x) The report shall include all conditions, procedures, and results. Data should be sufficiently detailed for an independent statistical analysis.

(xi) All treated birds should be sacrificed and disposed of properly. Control birds may be kept as breeding stock, but should not be used in any other tests. Control offspring may be reared and used in another test as adults.

(2)—(3) [Reserved]

(4) *Definitive test*—(i) *Test substance.* (A) The concentrations of test substance in the diet will be specified in the test rule. At least three treatment groups and a control group shall be used. The higher two treatment levels shall be multiples (often 5x, 10x, or 20x) of the lowest treatment level. The highest treatment levels shall usually be below lethal levels, unless predicted exposure levels are high enough to approximate lethal levels.

(B) The material to be tested shall be analytically pure and the degree of purity shall be reported along with the percentage of each impurity at levels specified in the test rule. If specifically required by a test rule for a particular substance or mixture, the technical grade shall be tested. The test rule will specify the degree of purity or a range of compositions of the technical grade material.

(ii) *Controls.* A concurrent control is required during every test. The control birds shall be from the same hatch as the test groups. Control and test birds shall be kept under the same experimental conditions. The test procedures shall be the same for control and treated birds, except that no test substance shall be added to the diets of control birds. If a carrier is used in preparation of the test diets, the same carrier shall be added to the diets of control birds in the highest concentration used for test diets. The use of shared controls is acceptable for concurrent tests as long as the same carrier is used for all the tests.

(iii) *Test groups and numbers of birds.* (A) Either one of two designs may be used for numbers of animals and pens. For one design, each of the three treatment groups and the control group shall consist of a minimum of 8 replicate pens, with each pen containing one male and three females. For the alternative design, each group shall consist of 12 replicate pens containing one male and one female per pen; the use of 20 replicate pens in the control group may yield a test with greater statistical power. Either design is acceptable as long as productivity reaches the definitive values given in paragraph (c)(4)(xii) of this section. Testing facilities using an experimental design with which they are not familiar are advised to experiment first without test substances in order to determine the feasibility of obtaining acceptable productivity levels.

(B) Test birds shall be impartially distributed among test chambers in such a manner that test results show no significant bias from the distributions.

(iv) *Duration of test.* (A) The test consists of three phases following acclimation to test facilities. the initial phase begins with exposure of treatment groups to diet containing the test substance and is typically 6 to 8 weeks long. After the initial phase, the photoperiod is manipulated according to paragraph (c)(4)(v) of this section to bring the hens into laying condition. This second phase ends with the onset of egglaying and is typically 2 to 4 weeks long. The final phase begins with the onset of laying and lasts for at least 8 weeks, preferably 10 weeks. A withdrawal study period may be added to the test phase if reduced reproduction is observed. The withdrawal period, if used, need not exceed 3 weeks.

(B) Exposure of adult birds to the test substance shall be continuous throughout the test. Unless otherwise specified in the test rule, test birds shall be exposed to the test substance for at least 10 weeks prior to the onset of egg laying.

(v) *Preparation for reproduction (photoperiod).* (A) Lighting regimes (photoperiod) are critical to successful reproduction. Various photoperiod regimes have been demonstrated to give acceptable results. Any photoperiod regime that results in productivity that meets the definitive values given in paragraph (c)(4)(xii) of this section is acceptable as long as birds are exposed to treated diets a minimum of 10 weeks prior to the onset of laying. Regardless of the method selected, lighting shall be controlled carefully. It is important during the initial phase to not interrupt the dark period unless absolutely necessary.

(B) A suggested photoperiod regime would consist of maintaining birds under a photoperiod of 7 or 8 hours of light during the initial phase. At the end of the initial phase, the photoperiod may be increased to 16-17 hours of light per day. The photoperiod may be maintained at this level for the remainder of the study, although an increase each week of 15 minutes per day is acceptable.

(vi) *Observations of record on adult birds.* (A) Body weights shall be recorded for each adult bird at the beginning of the treatment period, at 14-day intervals until the onset of egg laying, and at termination of treatment. Birds may be weighed during egg production phase of the study only if they are not unduly stressed by the procedure. Food consumption shall be measured and recorded by pen at least as often as body weights are measured prior to the onset of laying and at least bi-weekly throughout the rest of the study.

(B) Observations on adult birds shall be made at least once a day. Any mortality or other signs of toxicity shall be described and recorded by date or day of test. Gross pathological examinations shall be conducted on all birds that die during the test period, and for all survivors at the end of the test. Analysis for test substance residues of two or more tissues (e.g., muscle, fat) is encouraged, but not required unless specified in the individual Test Rule under Part 799 of this chapter.

(vii) *Egg collection, storage, and incubation.* All eggs shall be collected daily, marked according to the date collected and the parental pen from which collected, and should be stored at 16°C and 55 to 80 percent relative humidity. Storage in plastic bags may improve uniformity of hatching. Stored eggs should be turned daily. At biweekly intervals, eggs shall be removed for storage and be candled to detect eggshell cracks. Except for eggs with cracked shells and those eggs removed for eggshell thickness measurements, all eggs should be set after candling for incubation in a commercial incubator. If incubators are not equipped to automatically turn eggs, they should be turned daily by hand. During the incubation period, eggs should be maintained at 37.5°C and approximately 70-percent relative humidity. Eggs shall be candled again on day 14 of incubation to determine fertility and early death of embryos. A final candling shall be done on day 21 to measure embryo survival. On day 23, eggs shall be removed to a separate incubator or hatcher. Hatching will normally be complete by the end of day 27.

(viii) *Duckling maintenance.* By day 27 of incubation, the hatched mallard ducklings should be removed from the hatcher or incubator. Ducklings shall be either housed according to the appropriate parental pen group or individually marked (such as by leg bands) as to parental group and housed together. Ducklings should be maintained in commercial brooder pens or pens of similar construction. Pens should be constructed of galvanized metal or stainless steel. Temperature in the pens should be controlled, preferably by a thermostatic control device. A temperature gradient in the pen from approximately 35°C to approximately 22°C will allow young birds to seek a proper temperature. Temperature requirements for young birds typically decline over this range from birth through the first several weeks of life. Ducklings shall be provided a standard commercial duck starter ration, or its nutritional equivalent. No test substance may be added to the diets of ducklings. Ducklings shall be maintained until they are 14 days old.

(ix) *Observation of record on ducklings.* The hatchability, percentage of normal hatchlings, percentage of 14-day old survivors, and number of 14-day old survivors per hen shall be recorded and reported. Ducklings shall be observed daily from hatching until they are 14 days old. Mortality, signs of toxicity, and other clinical abnormalities shall be recorded at least cumulatively through day 5 and recorded by age from days 5 through 14. Average body weights shall be recorded for ducklings at day 14.

(x) *Eggshell thickness.* Once every two weeks all eggs newly laid that day shall be removed and measured for eggshell thickness. Eggs shall be opened at the girth (the widest portion), the contents washed out (or used or saved for egg residue analysis), and the shells air dried for at least 48 hours. The thickness of the shell plus the dried membrane shall be measured at a minimum of 3 points around the girth using a micrometer calibrated at least to 0.01 mm units.

(xi) *Typical observed values.* The values reported here represent those observed from a few testing facilities under their conditions. These values are not necessarily representative of those from all facilities, however, if a reproduction test does not meet or at least approach these values for control birds, then there is likely to be a problem with test procedures or conditions that should be investigated and corrected. Typical values include:

(A) *Eggs laid.* Normal values for mallards—28 to 38 eggs per hen per season.

(B) *Eggs cracked.* Normal values for mallards—0.6 to 6 percent of eggs laid.

(C) *Viable embryos (fertility).* Normal fertility values for mallards—85 to 98 percent of eggs set.

(D) *Live 21-day embryos.* Normal values for mallards—97 to 99 percent of viable embryos.

(E) *Hatchability.* Normal values for mallards—50 to 90 percent of viable embryos (fertile eggs).

(F) *14-day-old survivors.* Normal values for mallards—94 to 99 percent of eggs hatched.

(G) *Eggshell thickness.* Normal average values for mallards—0.34 to 0.39 mm.

(xii) *Definitive test criteria.* (A) A test is unacceptable if mallard duckling productivity in control groups does not average fourteen 14-day old survivors per hen over a 10 week period.

(B) A test is unacceptable if the average eggshell thickness in control groups is less than 0.34 mm.

(C) A test is unacceptable if more than 10 percent of the adult control birds die during the test.

(5) [Reserved]

(6) *Analytical measurements*—(i) *Statistical analysis.* (A) Experimental groups should be individually compared to the control group by analysis of variance. Other accepted statistical methods may be used as long as they are documented and described. In particular, regression analysis is highly desirable if the data and number of dose levels allow the use of this technique. Sample units are the individual pens within each treatment level or control. Analysis shall include:

(*1*) Body weights of adults.

(*2*) Food consumption of adults.

(*3*) Percentage of hens laying eggs. This should always be determined when pens contain a single pair; if feasible, it should be determined when pens contain groups.

(*4*) Number of eggs laid per pen.

(*5*) Percentage of cracked eggs.

(*6*) Percent viable embryos of eggs set.

(*7*) Percent live 21-day embryos of viable embryos.

(*8*) Percent hatching of viable embryos.

(*9*) Percentage of hatchlings that are normal.

(*10*) Percent 14-day-old survivors of normal hatchlings.

(*11*) Number of 14-day-old survivors per hen.

(*12*) Body weights of 14-day-old survivors.

(*13*) Eggshell thickness.

(B) [Reserved]

(ii) *Analysis for test substance concentrations.* (A) Samples of treated diets shall be analyzed to confirm proper dietary concentrations of the test substance. If samples cannot be analyzed immediately, they should be stored appropriately (e.g., frozen at a temperature of −15°C or lower) until analysis can be performed. Analyses shall be conducted on all test substance concentrations at the beginning of the test period and again 10 to 12 weeks later. If not otherwise available, data shall be generated to indicate whether or not the test substance degrades or volatilizes. If the test substance is known or found to be volatile or labile to the extent that 25 percent or more loss occurs within 1 week, then test substance diets shall be prepared (freshly or from frozen concentrate) at a frequency that will prevent more than 25 percent loss of test substance.

(B) The assay method used to determine actual concentrations shall be reported according to paragraph (e)(1)(vi) of this section.

(C) *Analysis of basal diet.* A nutrient analysis of the basal diet shall be included in the test report. For commercially prepared basal diets, the list of ingredients supplied by the manufacturer is normally sufficient, if it is detailed. The composition of any vitamin or other supplements should also be reported.

(d) *Test conditions*—(1) *Test species*—(i) *Selection.* (A) The mallard, *Anas platyrhynchos* L., is the test species. Test birds should be pen-reared. They may be reared in the laboratory or purchased from commercial breeders. Rearing stock and/or test birds shall be obtained only from sources that have met the requirements for "U.S. Pullorum-Typhoid Clean" classification under paragraph (f)(1) of this section. Birds shall be obtained only from sources whose colonies have known breeding histories. If possible, a history of rearing practices for test birds should be obtained and made available upon request. This history should include lighting practices during rearing, disease record, drug and any other medication administered, and exact age. Test birds shall be phenotypically indistinguishable (except for size) from wild stock. Conscientious breeders of such birds will periodically outbreed their flocks with genetically wild stock in order to maintain a genetic composition that approximates the heterogeneity of naturally occurring birds.

(B) All control and experimental birds used in a test shall be from the same source and strain. If shipped, all birds shall be examined following shipment for possible physical injury that may have occurred in transit. All birds should have a health observation period of at least 2 weeks prior to selection for treatment. Birds should be in apparent good health. Deformed, abnormal, sick, or injured birds shall not be used. A population of birds shall not be used if more than 3 percent of either sex die during the health observation period. Birds shall not have been selected in any way for resistance to toxic substances. Birds shall not have been used in a previous test, either in control or treatment group. Offspring of birds used in a treatment group in a previous test shall not be used, but offspring of birds used as controls in a previous test are acceptable.

(C) Test birds shall be approaching their first breeding season and shall be at least 7 months old. All test birds shall be the same age within 1 month. The age of test birds shall be reported.

(D) Mallards shall be acclimated to test facilities and untreated basal diet for at least 2 weeks. Acclimation may be in the actual pens used in the test or in identical pens. The acclimation period may coincide with the health observation period. Birds shall be randomly assigned to treatment and control pens. However, when birds in a pen are incompatible, they may be rearranged within a control or treatment group at any time prior to initiating treatment.

(E) During holding, acclimation, and testing, birds shall be shielded from excessive noise, activity, or other disturbance. Birds shall be handled only as much as is necessary to conform to test procedures.

(ii) *Diet*—(A) *Adult birds.* (*1*) A standard commercial duck breeder ration, or its nutritional equivalent, shall be used for diet preparation. This ration or basal diet shall be used for both control and treatment birds and shall be constant throughout the duration of the study. Antibiotics or other medication shall not be used in the diet or water of breeding birds. It may not be possible to obtain food that is completely free of pesticides, heavy metals, and other contaminants. However, diets shall be analyzed periodically for these substances and shall be selected to be as free from contaminants as possible. A nutrient analysis (quantitative list of ingredients) of the diet shall be included with the test report.

(*2*) The test substance shall be mixed into the diet in a manner that will ensure even distribution of the test substance throughout the diet. If possible, the test substance should be added to the diet without the use of a carrier or diluent. If a diluent is needed, the preferred diluent is distilled water; but water shall not be used for test substances known to hydrolyze readily. When a test substance is not water soluble, it may be dissolved in a reagent-grade evaporative diluent (e.g., acetone, methylene chloride) and then mixed with the test diet. The solvent should be completely evaporated prior to feeding. Other acceptable diluents may be used, if necessary, and include table-grade corn oil, propylene glycol, and gum arabic (acacia). If a diluent is used, it shall constitute no more than 2 percent by weight of the treated diet, and an equivalent amount of diluent shall be added to control diets.

(*3*) Diets may be mixed by commercial or mechanical food mixers. Other means are acceptable as long as they result in even distribution of the test substance throughout the diet. Screening of the basal diet before mixing is suggested to remove large particles. For many test substances, it is recommended that diets be mixed under a hood. Frequently, the test substance is added to an aliquot of the basal diet to form a premix concentrate. The premix concentrate should be stored so as to maintain the chemical concentration. For final preparation of test diets, the premix is mixed with additional basal diet to form the proper concentrations. The frequency with which final treated diets are prepared will depend upon the stability and other characteristics of the test substance. Unless otherwise specified in the test rule or determined by degradation or volatility studies, it is recommended that final diets be prepared weekly, either fresh or from a concentrate. For volatile or labile test substances, test diets shall be mixed frequently enough so that the concentrations are not reduced from initial concentrations by more than 25 percent. Analysis of diets for test substance concentrations is required as specified in paragraph (c)(6)(ii) of this section.

(*4*) Clean water shall be available *ad libitum.* Water bottles or automatic watering devices are recommended. If water pans or bowls are used, water shall be changed daily or more often.

(B) *Young birds.* Young birds produced during the test should be fed a commercial duck starter ration, or its nutritional equivalent. No test substance shall be added to the diets of young birds. No antibiotics or medication shall be used in the diet.

(2) *Facilities.* (i) Mallards should be housed in breeding pens or cages of adequate size conforming to good husbandry practices. Space requirements for mallards have not been well defined, but it is recommended that

there be at least 10,000 square centimeters (approximately 5.4 square feet) of floor space per bird. Documentation that reproductive parameters and health of birds are not adversely affected should be provided for cages much smaller than this area. The preferred construction materials are stainless steel, galvanized sheeting, and wire mesh. For enclosed cages, floors and external walls may be wire mesh; ceilings and common walls should be solid sheeting. Open-topped pens may be constructed of the same materials for the side walls with open tops and wire mesh or concrete floors. Concrete floors should be covered with litter such as straw, wood shavings, or sawdust. Other construction materials, except wood, are acceptable if they can be kept clean. Wood may be used as vertical framing posts for the support of wire mesh or for horizontal framing along the top of the pen. Wood should not be used for floors or lower sides of pens unless it has been coated with a non-adsorbent material such as perfluorocarbon plastic (e.g., Teflon) or unless the wood is replaced between tests.

(ii) Pens should be disassembled (if feasible) and should be cleaned thoroughly between tests. Steam cleaning of enclosed cages is recommended. Enclosed cages may be brushed thoroughly, as an alternative method. For open-topped pens, the sides and vertical supports should be thoroughly brushed. Any used floor litter should be discarded. The floor composition will dictate methods used to clean the floor. If litter is used on the floor, it should be fresh and clean when birds are placed in the pen. The use of detergents or bleach is acceptable, but other chemical disinfectants (such as quaternary ammonium compounds) should not be used. When necessary to control disease vectors, hot or cold sterilization techniques are recommended, as long as such techniques will not leave chemical residues on the cages. For cold sterilization, ethylene oxide is recommended.

(iii) Pens should be kept indoors in order to better control lighting, temperature, humidity, and other factors. Outdoor pens may be used only during the normal breeding season. The photoperiod should be carefully controlled, preferably by automatic timers. A 15 to 30 minute transition period is desirable. The photoperiod regime is described under paragraph (c)(4)(v) of this section. Lights shall emit a spectrum simulating that of daylight. The use of shorter wavelength "cool-white" fluorescent lights that do not emit the daylight spectrum should be avoided. Illumination intensity should be about 6 foot-candles at the level of the birds.

(iv) Temperature and humidity should be controlled during the study. Recommended levels are 21°C and 55 percent relative humidity. Temperature for indoor tests shall be recorded at least weekly at the same time of day and shall be reported. For tests conducted without temperature control, temperature minimums and maximums should be recorded daily. Continuous temperature monitoring is desirable. Temperature recording shall be made at levels of 2.5 to 4 cm above the floor of the cage. Recording of approximate humidity levels is also desirable. Good ventilation should be maintained. Suggested ventilation rates are 4 changes per hour in winter and 15 changes per hour in the summer.

(v) If facilities are being used for the first time, it may be desirable to allow birds to breed in the facility prior to testing in order to ensure that controls will have acceptable productivity according to the requirements given in paragraphs (c)(4) (xi) and (xii) of this section.

(e) *Reporting.* (1) The test report shall include the following information:

(i) Name of test, sponsor, test laboratory and location, principal investigator(s), and actual dates of beginning and end of test.

(ii) Name of species tested (including scientific name), age of birds (in months) at the beginning of the test, source of birds, and body weights for adult birds throughout the test.

(iii) Description of housing conditions, including type, size, and material of pen, temperature, humidity, photoperiod and lighting intensity, and any changes during the test.

(iv) Detailed description of the basal diet, including source, composition, diluents (if used), and supplements (if used). A nutrient analysis of the basal diet shall be included.

(v) Detailed description of the test substance including its chemical name(s), source, lot number, composition (identity of major ingredients and impurities), and known physical and chemical properties pertinent to the test (e.g., solubility, volatility, degradation rate, etc.).

(vi) The number of concentrations used, nominal and measured concentrations of test substance in each level, assay method used to determine actual concentrations, storage conditions and stability of treated diets, number of birds per pen and number of replicate pens per concentration and for controls.

(vii) Acclimation procedures and methods of assigning birds to test pens, including method of randomization, and any rearrangements due to incompatibility.

(viii) Frequency, duration, and methods of observation.

(ix) Description of any signs of intoxication, including time of onset, duration, severity (including death), and numbers affected, including accidental deaths or injuries.

(x) Food consumption per pen and any observations of repellency or food palatability.

(xi) Method of marking all birds and eggs.

(xii) Details of autopsies.

(xiii) Egg and hatching data in summary and by pen per week in sufficient detail to allow an independent statistical analysis. Data shall be presented for all of the parameters listed in paragraph (c)(6)(i) of this section. The number of eggs set also shall be reported.

(xiv) Egg storage, incubation, and hatching temperatures, relative humidities, and turning frequencies.

(xv) Observations of health and weights of young at 14 days of age.

(xvi) Location of all raw data storage.

(xvii) Methods of statistical analysis and interpretation of results.

(xviii) Anything unusual about the test, any deviation from these procedures, and any other relevant information.

(2) In addition, the following information shall be available upon request:

(i) A general description of the support facilities.

(ii) A description of the Quality Control/Quality Assurance program, including the Average Quality Level for the program element performing the test, procedures used, and documentations that these levels have been achieved.

(iii) The names, qualifications, and experience of personnel working in the program element performing the test, including the study director, principal investigator, quality assurance officer, as well as other personnel involved in the study.

(iv) Standard operating procedures for all phases of the test and equipment involved in the test.

(v) Sources of all supplies and equipment involved in the test.

(vi) Diagram of the test layout.

(vii) Originals or exact copies of all raw data generated in performing the test.

(viii) A detailed description, with references, of all statistical methods.

(f) *References.* For additional background information on this test guideline the following references should be consulted:

(1) U.S. Department of Agriculture. "National Poultry Improvement Plan," Report No. 2. *Directory of Participants Handling Waterfowl, Exhibition Poultry, and Game Birds.* U.S.D.A., Science and Education Administration, Beltsville, MD 20705 (1979).

(2) [Reserved]

[50 FR 39321, Sept. 27, 1985, as amended at 52 FR 19069, May 20, 1987]

## § 797.2175 Avian acute oral toxicity test.

(a) *Purpose.* The guideline in this section is designed to develop data on the acute oral toxicity to Northern bobwhite and Mallard for chemical substances and mixtures subject to acute environmental effects test regulations under the Toxic Substances Control Act (TSCA) (Pub. L. 94-469, 90 Stat. 2003 15 U.S.C. 2601 *et seq.*).

The Agency will use these and other data to assess the acute hazard to birds.

(b) *Definitions.* The definitions in section 3 of the Toxic Substances Control Act (TSCA) and Part 792—*Good Laboratory Practice Standards* of this chapter apply to this test guideline. In addition, the following definitions apply to this guideline:

(1) "Acclimation" Physiological or behavioral adaptation of test animals to environmental conditions and basal diet associated with the test procedure.

(2) "$LD_{50}$" The empirically derived dose of the test substance that is expected to result in mortality of 50 percent of a population of birds which is treated with a single oral dose under the conditions of the test.

(3) "Test substance" The specific form of a chemical or mixture of chemicals that is used to develop the data.

(4) "Observation period" The portion of the test that begins after the test birds have been dosed and extends at least 14 days.

(5) "Hatch" Eggs or birds that are the same age and that are derived from the same adult breeding population, where the adults are of the same strain and stock.

(c) *Test procedures*—(1) *Summary of test.* (i) After birds have been obtained, they are acclimated for at least 14 days. The dosage levels for the definitive test are established, possibly requiring a range-finding test to be conducted first. Test birds are randomly assigned to the various dosage levels and controls. Birds are weighed and the test substance is administered as a single oral dose either by gavage or capsule. Birds are closely monitored for 60 to 120 minutes after doses are given and then observed regularly for mortality or other signs of intoxication throughout the observation period. Birds are weighed and feed consumption is estimated at least weekly. The mortality pattern is examined and subjected to the appropriate statistical analysis to derive the $LD_{50}$, confidence limits, and slope of the dose-response line. The complete mortality pattern, along with signs of intoxication and necropsy data, should be reported.

(ii) A test is unacceptable if more than 10 percent of the control birds die during the test.

(2) [Reserved]

(3) *Range-finding test.* Unless the approximate toxicity of the test substance is known already, a range-finding test should be conducted to determine the dosage levels of the test substance to be used in the definitive test. Refer to paragraph (c)(4)(iv) of this section for details on dosage levels for definitive tests. Procedures for range-finding tests may vary, but generally, groups of a few birds are administered three to five widely-spaced doses. A series of 2, 20, 200, and 2,000 mg/kg-body weight is suggested. If a test substance is expected to be of low toxicity, it may be of advantage to conduct a limit test at 2,000 mg/kg first under paragraph (c)(4)(iv)(B) of this section. If mortality occurs at this level, then further range-finding at lower levels is suggested. The results of the range-finding test then may be used to establish the definitive test dosage levels.

(4) *Definitive test*—(i) *Administration of test substance.* (A) After acclimation under paragraph (d)(1)(i)(D) of this section, feed should be withheld from all test groups for a minimum of 15 hours prior to administration of the test substance. Dosing by gavage is preferred; where gavage is not feasible doses may be administered by gelatin capsule. Doses are to be based on the individual body weight of each bird; weights are typically determined at the time of dosing, but may be taken, especially for capsules, within 24 hours prior to dosing. Dosing should be done in the early morning hours.

(B) If a carrier is used to administer the test substance, the preferred carrier is distilled or deionized water unless the test substance is known to hydrolyze readily. Other acceptable carriers include corn oil, propylene glycol, and gum acacia. Materials with known toxic or emetic properties should not be used. The dosing volume of test substance plus carrier in a test should be constant for all birds with respect to individual body weights and should not exceed 5 ml/kg-body weight. For those unusual test substances that might require a larger dosing volume (e.g., liquids with low purity), a dosing volume up to 8 ml/kg may be used; however, the test species should be bobwhite or else steps should be taken to ensure that mallards do not regurgitate the dose.

(ii) *Controls.* (A) A concurrent control is required during every test. Control birds should be from the same hatch as the test groups. Control and test birds should be kept under the same experimental conditions. The test procedures should be the same for control and treated birds, except that no test substance should be administered to the control birds. Control birds should receive a sham dose consisting of the same carrier or capsule as received by the test birds. The use of shared controls is acceptable for concurrent tests as long as the same carrier or capsule is used for all the tests.

(B) A test is not acceptable if more than 10 percent of the control birds die during the test period.

(C) A concurrent positive control with a substance of known toxicity is not required. However, a quarterly or semi-annual test with a laboratory standard (reference toxicant) is recommended as a means of detecting possible interlaboratory or temporal variation. A laboratory standard is also recommended when there is any significant change in food, housing, or source of birds.

(iii) *Number and sex of animals tested.* (A) In the definitive test, a minimum of ten birds should be used for each dosage level of the test substance and for the control. Birds may be of either sex or both sexes in any ratio. A 1:1 ratio is commonly used.

(B) Birds at a dosage level may be divided into two pens of five birds each. If this is done, dividing the groups by sex is encouraged.

(iv) *Concentrations and dosage-mortality data.* (A) A minimum of five dosage levels of the test substance should be used in the definitive test. These levels should be spaced geometrically. The recommended spacing is for each dosage level to be at least 60 percent of the next higher level (less than 1.67 times the next lower level). Ideally, dosage levels should be spaced so that at least three levels should result in mortality between, but not including, 0 percent and 100 percent and at least one level should kill more than 50 percent and at least one level should kill less than 50 percent of the birds in a group. For some test substances, it may be necessary to use more than five dosage levels to achieve these results.

(B) For test substances expected to have relatively low toxicity, a limit test may be conducted at 2,000 mg/kg. The $LD_{50}$ may be reported as greater than 2,000 mg/kg if 10 birds are dosed at 2,000 mg/kg, if no mortality occurs, and if test procedures, number of controls, and duration are the same, except for the number of dosage levels, as in the definitive test. Signs of intoxication, if any, should be reported. No further testing is required at lower dosage levels.

(v) *Duration of test.* The definitive test consists of the administration of the test substance followed by an observation period of at least 14 days. If mortality occurs during the last 3 days of the 14-day period, or if signs of intoxication are not clearly in remission, or if the test substance is expected to have delayed effects, then the observation period should be extended to at least 21 days or until mortality or signs of intoxication are not observed for 72 hours.

(vi) *Observations.* (A) Birds should be monitored closely for the first 60 to 120 minutes after dosing. Any regurgitation should be noted and reported. Additional observations of test birds should be made, at a minimum, 3 times on the day of dosing and at least daily throughout the remainder of the test period. Where feasible, twice daily observations are recommended.

(B) Throughout the test period, all signs of intoxication, other abnormal behavior, and mortality should be recorded and reported by dosage level and by day. Signs of intoxication are those behaviors apparently due to the test chemical and may include a wide array of behaviors, such as labored respiration, leg weakness, hemorrhage, convulsions, ruffled feathers, etc. All signs of intoxication and any other abnormal behavior, such as excessive aggression, toe-picking, etc. that may or may not be attributed to the test substance should be reported. Among survivors, remission of signs of intoxication and cessation of abnormal behavior should be recorded by dosage level and by day. An estimate of the number of birds exhibiting such signs should be recorded for each dosage level.

(C) Individual body weights of birds should be recorded and reported for control and treated birds at the time of calculating the dosage to be administered and weekly thereafter until the test is concluded. An extra weighing the third day after dosing may provide useful information, especially on anorexia. Body weights of birds a week prior to dosing are not required, but would provide valuable base-line data. Feed consumption should be recorded at least weekly throughout the test; valuable additional information can be obtained by monitoring food consumption daily, especially for the first few days following dosing.

(D) Gross pathology examinations should be conducted on at least two or three birds dying at each dosage level and on all control birds that die. Gross pathological examinations of survivors are optional, but may provide valuable information, especialy for lesions associated with sublethal effects.

(5) [Reserved]

(6) *Analytical measurements*—(i) *Statistical analysis.* (A) The data should be analyzed, preferably by graphical or computational methods of probit analysis. The LD$_{50}$ value, 95 percent confidence limits, and slope of the transformed dose-response curve should be determined for mortality at the end of test. A test for heterogeneity of the data (e.g., chi square test) should be conducted. Other standard statistical methods are acceptable if they provide the slope of the dose-response line as well as the LD$_{50}$ value.

(B) All methods used for statistical analysis should be described completely.

(ii) *Analysis of basal diet.* (A) A proximate analysis of the basal diet should be included in the test report. The analysis should include percentages by weight of protein, fat, fiber, ash, calcium, and phosphorus. In addition to these analyzed components, a list of expected amounts of vitamins, minerals, or other supplements also should be reported. Most commercial feed companies provide both the analysis and the list of supplements on the label.

(B) A contaminants analysis of the feed should be conducted periodically for heavy metals (e.g., arsenic, cadmium, lead, mercury, and selenium) and persistent pesticides, especially chlorinated insecticides. A broader pesticide screen to include, for example, diazinon, methyl parathion, and malathion may be useful.

(d) *Test conditions*—(1) *Test species*—(i) *Selection.* (A) Northern bobwhite, *Colinus virginianus* (L.), and mallard, *Anas platyrhynchos* L., are the test species. Birds may be reared in the laboratory or purchased from a breeder. All control and treatment birds used in a test should be from the same source and breeding population. Birds should be obtained only from sources whose colonies have known breeding histories. Birds should be phenotypically indistinguishable (except for size) from wild stock. It is recommended that birds be obtained from flocks that have been outbred periodically in order to maintain a genetic composition that approximates the natural heterogeneity of the species.

(B) Birds used in the test should be in apparent good health. Deformed, abnormal, sick, or injured birds should not be used. Birds should not be used for a test if more than 5 percent of the total test population die during the 14-day acclimation period. Birds purchased from a breeder should be certified as disease-free or as bred from disease-free stocks. Birds should not have been selected in any way for genetic resistance to toxic substances. Birds should not have been used in a previous test, either in a treatment or control group.

(C) Test birds should be young adults, not yet mated, at least 16 weeks old at the time of dosing. A less preferred alternative is for the use of first year birds that may have been mated, as long as the birds are brought completely out of production through reduced light cycles. All birds used in a test should be the same age ±1 week. It is recommended that weights be at least 180g for bobwhite and 900g for mallard. More consistent responses may be attainable if the range of body weights is no greater ± 10 percent of the mean body weight for the test population. The age should be recorded and reported.

(D) Test birds should be acclimated to test facilities and basal diet for a minimum of 14 days. Acclimation to test pens should be in the actual pens used in the test. Birds used in the test should be assigned randomly to treatment and control pens, except that assignment may be made to result in only one sex per pen if replicate pens are used for each dosage level, under paragraph (c)(4)(iii) of this section. Randomization should be done at the initiation of the acclimation period.

(E) During holding, acclimation, and testing, birds should be shielded from excessive noise, activity, or other disturbance. Birds should be handled only as much as is necessary to conform to test procedures.

(ii) *Diet.* (A) A standard commercial game bird (for bobwhite) or duck (for mallard) feed or the nutritional equivalent, should be used as the diet. Feed should not be used past its normal shelf life. Antibiotics or other medication should not be used in the diet during the acclimation period or the test. It may not be possible to obtain feed that is completely free of pesticides, heavy metals, and other contaminants; however, diets should be analyzed periodically, under paragraph (c)(6)(ii)(B) of this section, and selected to be as free from contaminants as possible. Extra precautions should be taken when fish meal or oil is a major ingredient, since fish are often contaminated with high levels of chlorinated hydrocarbons.

(B) Clean water should be available *ad libitum.* Only clean, unmedicated water should be offered during the acclimation and testing periods. Water bottles or automatic watering devices are recommended. If water pans or bowls are used, water should be changed at least once a day.

(2) ***Facilities.*** (i) Tests should be conducted indoors with birds being maintained in commercial breeder or holding pens or pens of similar construction. Pens should be constructed of galvanized metal, stainless steel, or perfluorocarbon plastics. Materials that are toxic, likely to influence toxicity, or sorb test substances should not be used. Wire mesh should be used for floors and external walls; solid sheeting should be used for common walls and ceilings. Wire mesh for floors should be fine enough so as to not interfere with the normal movement of birds yet coarse enough to allow fecal material to fall through. Pens should have a floor area of at least 500 square centimeters per bird (approximately 75 square inches) for bobwhite and 1,000 square centimeters per bird (approximately 150 square inches) for mallards and should be at least 24 centimeters (approximately 9.5 inches) high for bobwhite and 32 centimeters (approximately 12.5 inches) high for mallard. Between tests pens should be disassembled (if feasible) and should be cleaned thoroughly. Steam cleaning of cages is recommended. Cages may be hosed, brushed thoroughly and hosed again, as an alternative method. The use of detergents or bleach is acceptable, but other chemical disinfectants such as quaternary ammonium compounds should not be used. When necessary to control disease vectors, hot or cold sterilization techniques are recommended, as long as such techniques will not leave chemical residues on the cages. For cold sterilization, ethylene oxide is recommended. Pens should not be cleaned during a test.

(ii) Testing is done indoors to control lighting and other environmental variables. Temperatures for adult birds should be maintained at normal indoor temperatures, preferably between 15 °C and 27 °C (60 to 80 °F). Ventilation should be sufficient to supply 10 to 15 air changes per hour. The test room should be maintained at a relative humidity of 45 to 70 percent. Higher humidities are appropriate for waterfowl. A photoperiod of 8 hours light and 16 hours dark is recommended in order to prevent birds from coming into reproductive condition. Lighting may be either incandescent of fluorescent. Pens and lights should be positioned so that all pens will receive approximately equal illumination.

(3) [Reserved]

(e) *Reporting.* (1) The report should include, but not necessarily be limited to, the following information:

(i) Name and address of the facility performing the study and the dates on which the study was initiated and was completed, terminated, or discontinued.

(ii) Objectives and procedures stated in the approved protocol, including any changes in the original protocol.

(iii) Statistical methods employed for analyzing the data.

(iv) The test and, if used, control substances identified by name, Chemical Abstracts Service (CAS) number or code number, source, lot or batch number, strength, purity, and composition or other appropriate characteristics.

(v) Stability of the test and, if used, control substances under the conditions of administration.

(vi) A description of the methods used, including:

(A) Description of housing conditions, including type, size, and material of pens, and the approximate test room temperature, humidity, ventilation rate, photoperiod, and lighting intensity.

(B) Detailed description of feed, including source, supplements (if used), and proximate analysis.

(C) Acclimation procedures and methods of assigning birds to test pens, and test pens to dose-levels.

(D) Frequency, duration, and methods of observations.

(vii) A description of the test system used, including the scientific name of the test species, number used, sex and reproductive history and condition, age (in weeks) at the beginning of the test, source, and procedures used for identification. Individual body weights (or means, extremes, and an estimate of variance) should be reported for the beginning of the test and weekly thereafter.

(viii) A description of the dosages, numbers of birds and replicates per dose, method and time of administration. The reported results should include:

(A) The results of range-finding tests, if conducted.

(B) For the definitive test, a description of signs of intoxication and other abnormal behavior, including time of onset, duration, severity (including death), and number affected at each dose level and control each day of the test.

(C) Feed consumption per pen at least weekly or as often as measured, if more frequently than weekly, along with an estimate of wastage.

(D) The results of gross pathological examinations.

(ix) A description of all circumstances that may have affected the quality or integrity of the data.

(x) The name of the sponsor, study director, principal investigator, names of other scientists or professionals, and the names of all supervisory personnel involved in the study.

(xi) A description of the transformations, calculations, or operations performed on the data, a summary and analysis of the data, and a statement of the conclusions drawn from the analysis. Results of the analysis of data should include the calculated $LD_{50}$ value, 95 percent confidence limits, slope of the transformed dose-response line, and the results of a goodness-of-fit test (e.g., chi-square test).

(xii) The signed and dated reports of each of the individual scientists or other professionals involved in the study, including each person who, at the request or direction of the testing facility or sponsor, conducted an analysis or evaluation of data or specimens from the study after data generation was completed.

(xiii) The locations where all specimens, raw data, and the final report are stored.

(xiv) The statement prepared and signed by the quality assurance unit.

### § 797.2750 Seed germination/root elongation toxicity test.

(a) *Purpose.* The guideline in this section is intended for use in developing data on the acute toxicity of chemical substances and mixtures ("chemicals") subject to environmental effects test regulations under the Toxic Substances Control Act (TSCA) (PUB. L. 94-469, 90 Stat. 2003, 15 U.S.C. 2601 *et seq.*). This guideline prescribes test procedures and conditions using seed of commercially important terrestrial plants to develop data on the phytotoxicity of chemicals. The United States Environmental Protection Agency (U.S. EPA) will use data from these tests in assessing the hazard of a chemical to the environment.

(b) *Definitions.* The definitions in section 3 of the Toxic Substances Control Act (TSCA) and the definitions in Part 792—*Good Laboratory Practice Standards* of this chapter apply to this test guideline. The following definitions also apply to this guideline:

(1) "ECX" means the experimentally derived chemical concentration that is calculated to effect X percent of the test criterion.

(2) "Embryo" means the young sporophytic plant before the start of germination.

(3) "Germination" means the resumption of active growth by an embryo. The primary root should attain a length of 5 mm for the seed to be counted as having germinated.

(4) "Hypocotyl" means that portion of the axis of an embryo or seedling situated between the cotyledons (seed leaves) and the radicle.

(5) "Radicle" means that portion of the plant embryo which develops into the primary root.

(6) "Test solution" means the test chemical and the dilution water in which the test chemical is dissolved or suspended.

(c) *Test procedures*—(1) *Summary of the test.* (i) Seed should be separated into appropriate size classes, and that size class containing the most seed used exclusively for the test. Fresh test solutions should be added to petri dishes that have been completely filled with either precleaned quartz sand, 200 micron glass beads, or other inert material. The seed should then be positioned on the substrate allowing adequate room for anticipated growth. It is recommended that the radicle end of the seed be aligned in the direction of this growth. Petri dish lids should be used to hold the seed in place, and the dishes sealed with tape. For those chemicals that are insoluble in water and that should be sorbed to the substrate, deionized or glass-distilled water should be added to the substrate prior to positioning the seed.

(ii) The dishes should be placed in a seed germinator or other growth facility at a slight angle to facilitate linear root growth. Seed should be incubated in the dark until at least 65 percent of the control seed have germinated and developed roots that are at least 20 mm long.

(iii) The number of seed that germinate should be counted, and root lengths measured. Concentration response curves, $EC_{10}$'s, and $EC_{50}$'s for seed germination and root elongation should be determined and reported for each of the species tested.

(2) *Chemical application.* (i) Test chemicals that are soluble in water should be dissolved in deionized or glass distilled water and added to the substrate in the petri dishes at the start of the test.

(ii) Test chemicals that are insoluble in water but which can be placed in aqueous suspension with a carrier should be suspended in deionized or glass-distilled water with the carrier and then added to the petri dishes. The carrier should be soluble in water, relatively nontoxic to plants, and should be used in the minimum amount required to dissolve or suspend the test chemical. There are no preferred carriers; however, acetone, gum arabic, polyethylene glycol, ethanol and others have extensively been used in testing herbicides, plant growth regulators, fungicides, and other chemicals that affect plants. Tests of the carrier effect should be included in the test experimental design and conducted simultaneously as controls.

(iii) Water-insoluble chemicals for which no nontoxic water-soluble carrier is available, should be dissolved in an appropriate volatile solvent. The solution and substrate should be placed in a rotary vacuum apparatus, and evaporated, leaving a uniform coating of test chemical on the substrate. A weighed portion of the substrate should be extracted with the same organic solvent and the chemical assayed before the containers are filled. Solvent controls should be included in the experimental design and tested simultaneously. Deionized or glass distilled water should be added to the treated substrate prior to positioning the seed on the substrate.

(3) *Range-Finding Test.* (i) A range-finding test should be conducted to establish (A) if definitive testing is necessary and (B) test solution concentrations for the definitive test.

(ii) The seed should be exposed to a chemical concentration series (e.g., 0.01, 0.1, 1.0, 10, 100, and 1,000 mg/l. The lowest concentration in the series, exclusive of controls, should be at the chemical's detection limit. The upper concentration, for water soluble compounds, should be the saturation concentration.

(iii) The test consists of one run for each of the recommended plant species or selected alternates. A minimum of 15 seed per species should be exposed to each chemical concentration and control. The test period may be ended when at least 65 percent of the control seed have germinated and developed roots that are at least 20 mm long. The exposure period may be shortened if data suitable to establish the test solution concentration series for the definitive test can be obtained in less time and if the definitive test is to be conducted. No replicates are required; and nominal concentrations of the chemical are acceptable unless definitive testing is not required as specified in paragraph (c)(3)(iv) of this section.

(iv) Definitive testing is not necessary if the highest chemical concentration tested results in less than a 50 percent inhibition of germination or reduction in root growth or if the lowest concentration tested (analytical detection limit) results in greater than a 50 percent inhibition of germination or reduction in growth.

(v) Graphical analysis of the range-finding data facilitates selection of chemical concentrations for the definitive test.

(4) *Definitive test.* (i) The purpose of the definitive test is to determine the concentration-response curves, the $EC_{10}$'s and $EC_{90}$'s for seed germination and root elongation for each species tested, with the minimum amount of testing beyond the range-finding test.

(ii) The seed of each species tested should be exposed to at least 6 concentrations of the chemical chosen in a geometric series in which the ratio is between 1.5 and 2.0 (e.g., 2,4,8,16,32, and 64 mg/l). The concentration ranges should be selected to determine the concentration response curves between the $EC_{10}$ and $EC_{90}$ for both germination and root elongation. Test solutions or substrate extracts should be analyzed to determine chemical concentration prior to use. Selection of seed from the size class lot to be exposed to each test concentration should be unbiased.

(iii) At least three replicates, each with at least 10 seed per species should be tested for each concentration and control.

(iv) Every test should include controls consisting of the same dilution water, conditions, procedures and seed from the same lot used in the exposure group, except that none of the chemical is added. If a carrier (solvent) is needed to suspend or disperse the chemical, a separate carrier control should also be used.

(v) The test period may be ended when at least 65 percent of the control seed have germinated and developed roots that are at least 20 mm long. When both conditions are satisfied, the mean number of seed germinating and mean root length per treatment (and control) can be determined. If the test chemical concentration series does not bracket the $EC_{10}$ through $EC_{90}$, for both germination and root elongation, the test should be repeated (at a higher or lower concentration series). Concentration response curves, $EC_{10}$'s and $EC_{90}$'s for germination and root elongation should be determined for each species tested and reported along with their 95 percent confidence limits.

(vi) Any abnormal seedling development or appearance such as lesions, enhanced root growth (measured), discoloration, swelling, loss of turgor, etc., should also be reported.

(vii) A randomized complete block design is recommended for the definitive test with blocks delineated within the seed germinator or growth chamber. If, for any reason, blocking is not feasible total randomization within chambers is acceptable.

(viii) Temperature in the germination facility should be recorded hourly. The pH of the test solutions should be recorded at the initiation of the definitive test.

(5) [Reserved]

(6) *Analytical measurements*—(i) *Test chemical.* Stock solutions should be diluted with glass distilled or deionized water to obtain the test solutions. Standard analytical methods, if available, should be used to establish concentrations of these solutions and should be validated before beginning the test. An analytical method is not acceptable if likely degradation products of the chemical, such as hydrolysis and oxidation products, give positive or negative interference. The pH of these solutions should also be measured prior to use.

(ii) *Numerical.* The number of seeds that germinate shall be counted and root lengths measured for each definitive test species. All root elongation measurements for a given species should be made sequentially before proceeding to the next species. Root length should be measured from the transition point between the hypocotyl and root to the tip of the root. Means and standard deviations should be calculated and plotted for each treatment and control. Appropriate statistical analyses should provide a goodness-of-fit determination for the concentration response curves.

(d) *Test conditions*—(1) *Test species.* (i) Test plants recommended for use include:

(A) *Lycopersicon esculentum* (tomato).

(B) *Cucumis sativus* (cucumber).

(C) *Lactuca sativa* (lettuce).

(D) *Glycine max* (soybean).

(E) *Brassica oleracea* (cabbage).

(F) *Avena sativa* (oat).

(G) *Lolium perenne* (perennial ryegrass).

(H) *Allium cepa* (common onion).

(I) *Daucus carota* (carrot).

(J) *Zea mays* (corn).

(ii) Other species of economic or ecological importance to the region of impact, may also be appropriate for testing. A minimum of 10 species should be tested.

(iii) Information on seed lot, the seed year or growing season collected, and germination percentage should be provided by the supplier of the seed. Only untreated seed (not treated with fungicides, repellents, etc.) taken from the same lot, and year or season of collection should be used in a given test. In addition, all seed of a species used in a test should be from the size class which contains the most seed. Damaged seed should be discarded. Standard seed dockage sieves should be used to size seed.

(2) *Facilities*—(i) *Apparatus.* (A) seed germinator, or other controlled environment chamber capable of maintaining a uniform testing temperature of 25 ± 1 °C is required. In addition, the facilities should include work areas for sizing, counting, and exposing seed for root measurement. If possible, these areas should be isolated from other activities. A fume hood may be needed when testing substances potentially hazardous to human health. Apparatus for distilling and deionizing water are needed unless reagent grade water is used. Refrigeration facilities to hold the seed in cold storage (5 °C) in moisture-proof containers at seed moisture contents of less than 10 percent are also needed.

(B) Disposal facilities should be adequate to accommodate spent glassware, sand, beads, and test solutions at the end of each run and any bench covering, lab clothing, or other contaminated materials.

(ii) *Containers and support media.* A minimum of 210 petri dishes and sufficient sand or glass beads, or other inert substrate to fill them are needed. Large (200 mm) glass petri dishes are recommended. Perlite, vermiculite, or native soils, should not be used as substrates.

(iii) *Cleaning and sterilization.* (A) All glassware and the substrate should be cleaned following standard good laboratory practice before each test. The substrate should be washed in half strength concentrated nitric acid and rinsed with a mild base followed by washes of glass-distilled or deionized water. The pH of the washed substrate should be near neutral. If the glass beads are to be reused, they should be heated to 100 °C for 8 to 12 hours prior to acid washing. A dichromate solution should not be used for cleaning beads or petri dishes. The sand and plastic petri dishes should not be reused.

(B) If fungal or other microbial contamination interferes with seed germination such that germination is less than 65 percent in the controls, glassware should be sterilized and/or the seed surface sterilized prior to use, e.g., the seed may be soaked for 10 minutes in a 10 percent sodium hypochlorite solution, then rinsed and soaked for 1 hour in glass-distilled water.

(3) *Test parameters.* Environmental conditions should be controlled to maintain incubation temperature at 25± 1 °C in complete darkness. If species other than the ten recommended for use are tested, incubation conditions may have to be adjusted to meet germination and root length criteria in the controls

(e) *Reporting.* The sponsor should submit to the USEPA all data developed during the test that are suggestive or predictive of phytotoxicity. In addition to the general reporting requirements prescribed in Part 792—*Good Laboratory Practice Standards* of this chapter, the following should be reported:

(1) Information on the source and history of the seed, germination percentage reported by the supplier, and the seed size class used for testing.

(2) The number of seed of each species per treatment, the number of replicates, carriers, incubation conditions, and seed sterilization procedures.

(3) The concentration of the chemical added to each treatment dish and its pH (pH is optional).

(4) If the range-finding test showed that the highest concentration of the chemical tested (not less than 1,000 mg/l) had no effect on the test species, report the results by species and concentration and a statement that the chemical is of minimum phytotoxic concern.

(5) If the range-finding test showed greater than 50 percent inhibition of germination or root elongation at a test concentration at the analytical detection limit, the results by species and concentration and a statement that the chemical is phytotoxic below the analytical detection limit.

(6) For each species included in the definitive test, means and standard deviations for germination and root length in each treatment. In addition, concentration response curves with 95 percent confidence limits delineated, goodness-of-fit determination, and $EC_{10}$'s and $EC_{50}$'s identified.

(7) Methods and data records of all chemical and numerical analyses including method validation and reagent blanks.

(8) The data records of the incubation temperature, germination counts, and root length measurements.

## § 797.2800 Early seedling growth toxicity test.

(a) *Purpose.* The guideline in this section is intended for use in developing data on the toxicity of chemical substances and mixtures ("chemicals") subject to environmental effects test regulations under the Toxic Substances Control Act (TSCA) (Pub. L. 94-469, 90 Stat. 2003, 14 U.S.C. 2601 *et seq.*). This guideline prescribes tests using commercially important terrestrial plants to develop data on the phytotoxicity of chemicals. The United States Environmental Protection Agency (U.S. EPA) will use data from these tests in assessing the hazard of a chemical to the environment.

(b) *Definitions.* The definitions in section 3 of the Toxic Substances Control Act (TSCA), and Part 792—*Good Laboratory Practice Standards* of this chapter apply to this test guideline. The following definitions also apply to this test guideline.

(1) "EC X" means the experimentally derived chemical concentration that is calculated to effect X percent of the test criterion.

(2) "Germination" means the resumption of active growth by an embryo.

(3) "Support media" means the quartz sand or glass beads used to support the plant.

(c) *Test Procedures*—(1) *Summary of the Test*—(i) *Root exposure.* In preparation for the test, seeds are planted in the potting containers (or in cotton or glass-wool plugs supported in hydroponic solution) and after germination seedlings are thinned by pinching the stem at the support medium surface to the 10 most uniform seedlings per pot. This marks the start of the test and the time of first application of test chemical. Seedlings emerging after this time are also pinched off at the surface. Potting mixtures of sand or glass beads are subirrigated with nutrient solution. Chemicals are applied to the plants via nutrient solution or are adsorbed to the support media. Plants are harvested after 14 days and analyzed for growth.

(ii) *Foliar exposure.* The foliar exposure test is identical to the root exposure test except that chemicals are applied to plants by either spraying or dusting the foliage or by exposing the plants to gas in a fumigation chamber.

(2) *Chemical application*—(i) *Root exposure.* (A) Chemicals that are soluble in water should be dissolved in the nutrient solution just prior to the beginning of the test. Deionized or glass-distilled water should be used in making stock solutions of the test chemical. Sufficient quantities of each concentration should be made up as needed to minimize storage time and disposal volume.

(B) Chemicals that are insoluble in water, but which can be suspended in an aqueous solution by a carrier, should be added, with the carrier, to the nutrient solution. The carrier should be soluble in water, relatively nontoxic to plants, and should be used in the minimum amount required to dissolve or suspend the test chemical. There are no preferred carriers; however, acetone, gum arabic, polyethylene glycol, ethanol, and others have extensively been used in testing herbicides, plant growth regulators, fungicides, and other chemicals that affect plants. Carrier controls should be included in the experimental design of the test and tested simultaneously.

(C) Water-insoluble chemicals for which no nontoxic, water-soluble carrier is available, should be dissolved in an appropriate volatile solvent. The solution should be mixed with the sand or glass beads which are then placed in a rotary vacuum apparatus and evaporated leaving a uniform coating of chemical on the sand or beads. A weighed portion of beads should be extracted with the same organic solvent and the chemical assayed before the potting containers are filled. Solvent controls should be included in the experimental design and tested simultaneously.

(ii) *Foliar exposure.* (A) Water-soluble chemicals should be dissolved in deionized or glass-distilled water just prior to use. Sufficient quantities of each concentration should be made up as needed. These solutions should be applied daily (during the normal 5-day work week). Plants should be placed in an exhaust hood and the chemical applied to the foliage. A plastic sleeve may be fitted over the top of the pot, and the foliage sprayed with specific quantities of test solution at known concentrations. The plastic sleeve, confining the chemical to plant and pot, facilitates expression of chemical dosage to quantity per pot area (i.e., $\mu g/m^2$). Shoots of control plants should be sprayed in an identical manner with deionized or distilled water. Alternatively, a miniature compressed-air sprayer mounted on a pendulum and equipped to automatically spray a plant positioned directly beneath the center of its arc of swing may be used.

(B) Water-insoluble chemicals, existing as solids, may be prepared for testing by grinding or other reduction to particles of <200 um diameter. Each day (during the normal 5-day work week) plants should be placed in an exhaust hood, a plastic sleeve fitted over the top of the pot, and specific quantities of chemical sprinkled uniformly over the potted seedlings. Prior to chemical application, plants should be misted with water to promote foliar retention of the chemical. Control plants should also be misted with deionized or distilled water at each treatment date and dusted with an inert material of the same particle size. Applications are expressed as quantity per unit pot area (i.e., $\mu g/m^2$).

(C) Chemicals existing in gaseous form at normal ambient temperatures and pressures can be generated as needed or stored under pressure. The bottled gas may be 100 percent chemical or may be mixed with an inert carrier, such as nitrogen, to known concentrations. Chemicals of controlled or measured concentrations should be metered into the exposure chamber, uniformly mixed about the plants, and exhausted through an outlet port.

(3) *Range-finding test.* (i) A range-finding test should be conducted to establish (A) if definitive testing is necessary and (B) the concentrations of test substance used in the definitive test for each species.

(ii) The recommended procedure is to expose newly germinated seedlings to a series of widely spaced concentrations of test chemical and assess effect as growth reduction. Seeds (approximately 30) should be planted directly in containers filled to within 2.5 cm of the top with quartz sand or glass beads. If a hydroponic system is used, the seeds should be planted in plugs of cotton or glass wool supported at the top of the solution. When 50 percent of the seeds have germinated the seedlings should be thinned (by pinching) to the 10 most uniform per pot and exposed to a widely spaced concentration series (i.e., 0.01, 0.1, 1.0, 10, 100, 1,000 mg/l) of test chemical. The lowest concentration in the series, exclusive of controls, should be at the chemical's detection limit. The upper concentration, for water-soluble compounds, should be the saturation concentration. If the anticipated fate of the chemical is soil or soil water, and the mechanism of concern is root uptake, the chemical should be applied in nutrient solution to the root support media (or coated on sand or glass beads for non-water soluble chemicals). With a chemical whose anticipated mode of exposure to plants is surface deposition by atmospheric transport, or irrigation water, the appropriate testing method may be foliar application allowing subsequent movement into the rooting zone with watering. Effect is assessed as growth reduction.

(iii) Alternatively, the seed germination/root elongation test may be used to establish the appropriate concentration range for testing.

(iv) No replicates are required and nominal concentrations are acceptable unless definitive testing is not required.

(v) Definitive testing is not necessary if the highest chemical concentration tested results in less than a 50 percent reduction in growth or if the lowest concentration tested (analytical detection limit) results in greater than a 50 percent reduction in growth.

(4) *Definitive test.* (i) The purpose of the definitive test is to determine the concentration response curves and the $EC_{10}$'s and $EC_{50}$'s for each of the species tested with the minimum amount of testing beyond the range-finding test.

(ii) At least 5 concentrations of chemical, exclusive of controls, should be used in the definitive test. For each species tested the concentration range should be selected to define the concentration-response curve between the $EC_{10}$ and $EC_{50}$'s. Test chemicals should be added to the hydroponic or nutrient solution or coated on the support media for the root exposure test; or sprayed, dusted, or gassed directly on the foliage in the foliage exposure tests.

(iii) Control pots should be included in the experimental design and should be used in each run. In addition, a carrier control should also be used for those chemicals that need to be solubilized.

(iv) If plants are to be grown hydroponically, seeds should be planted in plugs of cotton or glass wool supported in the tops of the containers. When sand or glass beads are used, the recommended planting procedure is to fill the potting containers to within 2.5 cm of the top and to sow seeds directly on the support media. After 50 percent of the seeds have germinated, the seedlings should be thinned to the 10 most uniform per pot.

(v) Alternative planting methods may be required when the chemical is highly volatile. An impervious barrier of polyethylene film, a modification of the double pot method, a glass plate, or other appropriate apparatus should be used to prevent volatilization from the root zone. Seeds should be germinated in the dark at 25 °C and seedlings with radicle lengths in the median range transplanted into the potting containers. The seedlings should be positioned such that their roots are exposed to the support media while the shoots pass through holes in the barrier. A ring of nontoxic, inert, pliable putty should be used to seal the holes around the stems. Control pots should be handled identically to the test pots except there is no exposure to the test chemical. This transplanting procedure, without the volatilization barrier, is also recommended when the test chemical is adsorbed to the support medium.

(vi) The test consists of one run for each of the recommended plant species or selected alternates. The duration of a run should be at least 14 days from the time that 50 percent of the seeds have germinated. For a particular chemical, a run is defined as exposure of the plant species to five concentrations of the chemical in a minimum of 3 replicate pots (10 plants per pot), with appropriate controls, followed by weight and height determinations and analysis.

(vii) All abnormalities (visible effects of the chemicals on plant growth and morphology including stunting of growth, discoloration, chlorosis and/or necrosis of the leaves, or morphological abnormalities) should be recorded. Observations of plants should be made daily (during the normal 5-day work week).

(viii) A randomized complete block design is recommended for this test with blocks delineated within the chambers or over greenhouse benches and randomization of treatment occurring within the blocks. If, because of very large pots, there exists inadequate space within chambers for blocking, total randomization within chambers is acceptable.

(ix) Irradiation measurements should be taken at the top of the plant canopy and the mean, plus a maximum and a minimum value, determined over the plant-growing area. These measurements should be taken daily and should be taken at least at the start and finish of the test. If the test is conducted in a greenhouse facility, hourly measurements of irradiation should be recorded and presented as daily total irradiance plus representative hourly curves for clear sky conditions and cloudy days.

(x) Temperature and humidity should be measured daily at the top of the plant canopy during each light and dark period.

(xi) Measurements of carbon dioxide concentration should be made at the top of the plant canopy (of chamber-grown plants) on a "continuous basis."

(5) [Reserved]

(6) *Analytical measurements*—(i) *Chemical.* Stock solutions should be diluted with glass distilled or deionized water to obtain the test solutions. Standard analytical methods, if available, should be used to establish concentrations of these solutions and should be validated before beginning the test. An analytical method is not acceptable if likely degradation products of the chemical, such as hydrolysis and oxidation products, give positive or negative interference. The pH of these solutions should also be measured prior to use.

(ii) *Numerical.* Mass and length of roots, shoots, and entire plants (root and shoot) should be measured for the definitive test. Means and standard deviations should be calculated and plotted for each treatment and control. Appropriate statistical analyses should provide a goodness-of-fit determination for the concentration-response curves.

(d) *Test conditions*—(1) *Test Species*—(i) *Selection.* (A) Test plants recommended for the definitive test include:

(*1*) *Lycopersicon esculentum* (tomato).

(*2*) *Cucumis sativus* (cucumber).

(*3*) *Lactuca sativa* (lettuce).

(*4*) *Glycine max* (soybean).

(*5*) *Brassica oleracea* (cabbage).

(*6*) *Avena sativa* (oat).

(*7*) *Lolium perenne* (perennial ryegrass).

(*8*) *Allium cepa* (common onion).

(*9*) *Daucus carota* (carrot).

(*10*) *Zea mays* (corn).

(B) Other species, of economic or ecologic importance to the region of impact, may also be appropriate and selected for testing.

(ii) *Seed selection.* Information on seed lot, the seed year or growing season collected and germination percentage should be provided by the source of the seed. Only untreated seed (not treated with fungicides, repellants, etc.) taken from the same lot, and year or season of collection should be used in a given test. In addition, all seed of a species used in a test should be of the same size class; and that size class which contains the most seed should be selected and used in a given test. Any seed which is damaged should be discarded.

(2) *Facilities*—(i) *Apparatus*. (A) Greenhouses or environmental chambers should provide adequate environmental controls to meet the carbon dioxide, humidity, irradiation, photoperiod, and temperature specifications. Chambers should be designed to prevent escape of internal air into the external environment other than through appropriate filtering material or media to prevent contamination of the external environment with the test chemical.

(B) Laboratory facilities for chemical determinations should include nonporous floor covering, absorbent bench covering with nonporous backing, and adequate disposal facilities to accommodate plant nutrient, test and wash solutions containing test chemicals at the end of each run, and any bench covering, lab clothing, or other contaminated materials.

(ii) *Containers and support media*. For each run, 18 polyethylene pots sufficiently large to grow at least 10 plants up to 14 days, are required for each species. It is equally acceptable to use small, individual containers if plants are grown in hydroponic solution. An additional 3 pots will be needed if a carrier control is needed. Potting containers used in each experiment should be of equal size and volume and possess the same configuration. When sand or glass beads are used, the potting containers should be filled to within 2.5 cm of their tops. Perlite, vermiculite, native soils, etc., should not be used for root support.

(iii) *Cleaning and sterilization*. (A) Potting and receiving containers, nutrient storage containers, and root support medium should be cleaned before use. All equipment should be washed according to good standard laboratory procedures to remove any residues remaining from manufacturing or prior use. A dichromate solution should not be used for cleaning beads or pots.

(B) Rooting media other than glass beads should be discarded at the end of the experiment. Disposal should conform to existing regulations.

(iv) *Nutrient media*. Half-strength modified Hoagland nutrient solution should be utilized as nutrient media for this test. When sand or glass beads are used as a support media, the potting containers should be filled with nutrient solution and drained periodically. An automated system design is recommended.

(3) *Test parameters*. Environmental conditions should be maintained as specified in this paragraph.

(i) Carbon dioxide concentration at $350 \pm 50$ ppm.

(ii) Relative humidity should approach $70 \pm 5$ percent during light periods and 90 percent during dark periods.

(iii) Irradiation, measured at 1 meter from the source, at $350 \pm 50\ \mu E/m^2$ sec at 400 to 700 nm.

(iv) Photoperiods of 16 hours light and 8 hours darkness.

(v) Day/night temperatures at $25^\circ/20^\circ \pm 3^\circ C$.

(e) *Reporting*. Reporting requirements of Part 792—*Good Laboratory Practice Standards* of this chapter apply to this guideline. The following data should be reported for each of the species tested in tabular form:

(1) Concentration of chemical in nutrient solution and in the root support material when the chemical is soluble in water or solubilized with a carrier compound; or the concentration of carrier compound in nutrient solution when carrier is used; or the quantity of chemical per unit weight of root support material when it is coated on the material.

(2) The quantity of chemical, the concentration at which it was applied, and the number of applications for those chemicals applied to the foliage.

(3) Environmental conditions (day/night temperatures, relative humidity, light intensity, carbon dioxide concentration, and photoperiod).

(4) Mass of above ground (shoot) and below ground (root) portion of each plant and mass of each whole plant (dry weight at 70 °C).

(5) Length of shoot, root, and entire plant.

(6) Visible effects of chemical, if any, on the intact plants.

(7) Means and standard deviations for mass and length of roots, shoots, and entire plants in each treatment and control. In addition, concentration- response curves with 95 percent confidence limits delineated, goodness-of-fit determination, and $EC_{10}$'s and $EC_{50}$'s identified.

### § 797.2850 Plant uptake and translocation test.

(a) *Purpose.* The guideline in this section is intended for use in developing data on the uptake and translocation of chemical substances and mixtures ("chemicals") by terrestrial plants subject to environmental effects test regulations under the Toxic Substances Control Act (TSCA) (Pub. L. 94-469, 90 Stat. 2003, 14 U.S.C. 2601 *et seq.*). This guideline prescribes tests using commercially important terrestrial plants to develop data on the quantity of chemical substances incorporated in plant tissues and the potential for entry into food chains with resultant indirect human exposure. The United States Environmental Protection Agency (U.S. EPA) will use data from these tests in assessing the hazard of a chemical to the environment.

(b) *Definitions.* The definitions in section 3 of the Toxic Substances Control Act (TSCA), and Part 792—*Good Laboratory Practice Standards* of this chapter apply to this test guideline. The following definitions also apply to this guideline:

(1) "EC X" means the experimentally derived chemical concentration that is calculated to effect X percent of the test criterion.

(2) "Mass balance" means a quantitative accounting of the distributions of chemical in plant components, support medium, and test solutions. It also means a quantitative determination of uptake as the difference between the quantity of gas entering an exposure chamber, the quantity leaving the chamber, and the quantity adsorbed to the chamber walls.

(3) "Support media" means the sand or glass beads used to support the plant.

(4) "Translocation" means the transference or transport of chemical from the site of uptake to other plant components.

(c) *Test procedures*—(1) *Summary of the test*—(i) *Root exposure.* In preparation for the test, seeds are planted in the potting containers (or in cotton or glass-wool plugs supported in hydroponic solution) and, after germination, seedlings thinned, by pinching the stem at the support surface. Potting mixtures of sand or glass beads should be subirrigated with nutrient solution. Chemicals are applied to the plants via nutrient solution or adsorbed to the support media. Carrot, lettuce, onion, cabbage, and ryegrass may be harvested whenever there is adequate plant material for chemical analysis. Cucumber, corn, soybean, tomato, and oats should be grown until fruit or seed are mature.

(ii) *Foliar exposure.* The foliar exposure test is identical to the root exposure test except that chemicals are applied to plants by either spraying or dusting the foliage or exposing the plants to gas in a fumigation chamber. If plants are fumigated, either rates of uptake and surface adsorption should be calculated, or the plants may be harvested and analyzed for test chemical and residues.

(2) *Chemical Application*—(i) *Root exposure.* (A) Chemicals that are soluble in water should be dissolved in the nutrient solution just prior to the beginning of the test. Deionized or glass distilled water should be used in making stock solutions of the test chemical. Sufficient quantities of each concentration should be made up as needed to minimize storage time and disposal volume.

(B) Chemicals that are insoluble in water but which can be placed in aqueous suspension with a carrier should be added, with the carrier, to the nutrient solution. The carrier should be soluble in water, relatively nontoxic to plants, and should be used in the minimum amount required to dissolve or suspend the test chemical. There are no preferred carriers; however, acetone, gum arabic, polyethylene glycol, ethanol, and others have been used extensively in testing herbicides, plant growth regulators, fungicides, and other chemicals that affect plants. Carrier controls shoud be included in the experimental design and tested simultaneously.

(C) Water-insoluble chemicals for which no nontoxic, water-soluble carrier is available, should be dissolved in an appropriate volatile solvent. The solution should be mixed with the sand or glass beads which are then placed in a rotary vacuum apparatus and evaporated, leaving a uniform coating of chemical on the sand or beads. A weighed portion of beads should be extracted with the same organic solvent and the chemical assayed before the potting containers are filled. Solvent controls should be included in the experimental design and tested simultaneously.

(ii) *Foliar exposure.* (A) Water soluble chemicals should be dissolved in deionized or glass distilled water just prior to use. Sufficient quantities of each concentration should be made up as needed. These solutions should be applied at weekly intervals. Plants should be placed in an exhaust hood and the chemical applied to the foliage. A plastic sleeve may be fitted over the top of the pot, and the foliage sprayed with specific quantities of test solution at known concentrations. The plastic sleeve, confining the chemical to plant and pot, facilitates expression of chemical dosage as quantity per pot area (i.e., $\mu g/m^2$). Shoots of control plants should be sprayed in an identical manner with deionized or distilled water. Alternatively, a miniature compressed-air sprayer may be mounted on a pendulum and equipped to automatically spray a plant positioned directly beneath the center of its arc of swing. When radioisotope-labelled chemicals are applied, health and safety considerations prohibit spray application. Instead, specific quantities of labelled chemical should be applied directly to leaves in single drops.

(B) Water-insoluble chemicals, existing as solids, may be prepared for testing by grinding or other reduction to particles of <200 um diameter. These chemicals should be applied at weekly intervals. Plants should be placed in an exhaust hood, a plastic sleeve fitted over the top of the pot, and a specific quantity of chemical sprinkled uniformly over them. Prior to chemical application, plants should be misted with water to promote foliar retention of the chemical. Control plants also should be misted with deionized or distilled water at each treatment date and dusted with an inert material of the same particle size. Applications should be expressed as quantity per unit pot area (i.e., $\mu g/m^2$).

(C) Chemicals existing in gaseous form at normal ambient temperatures and pressures should be generated for use as needed or stored under pressure. The bottled gas may be 100 percent pure chemical or mixed with an inert carrier, such as nitrogen, to known concentrations. Chemicals of controlled or measured concentrations should be metered into the exposure chamber, uniformly mixed about the plants, and exhausted through the outlet port where the flow rate and concentration are again measured. Use of this systems design provides an alternate method of analysis if the quantity of chemical sorbed by plants is less than that required for chemical analysis. Plants should be fumigated whenever they have reached sufficient size for measurement of photosynthesis and transpiration rates, assuming equivalent detection sensitivity of carbon dioxide, water vapor, and chemical analyzers. The appropriate size is a function of the gas exchange system and constitutes an area of expert judgment.

(3) *Range-finding test.* (i) A range-finding test should be conducted to establish the chemical concentrations used in the uptake and translocation test.

(ii) Because of the different mechanisms involved in root and leaf uptake, and to more closely define the chemical concentrations to be used in the uptake test, the definitive early seedling growth test is recommended as the range-finding test. Seeds should be germinated directly in containers filled with sand or glass beads or in cotton or glass-wool plugs supported in hydroponic solution. When 50 percent of the seedlings have germinated, the seedlings should be thinned (by pinching) to the 10 most uniform per container and exposed to a concentration series of test chemical. The lowest concentration in the series, exclusive of controls, should be at or below the $EC_{10}$ while the upper concentration should be at or above the $EC_{90}$. If the anticipated fate of the chemical is soil or soil-water, and the mechanism of concern is root uptake, the chemical should be applied in nutrient solution to the root support media (or coated on sand or glass beads for non-water soluble chemicals). With a chemical whose anticipated mode of exposure to plants is surface deposition by atmospheric transport or irrigation water, the appropriate testing method may be foliar application allowing subsequent movement into the rooting zone with watering. Effect is assessed as growth reduction. The concentration selected as the upper limit for the uptake and translocation test should be near the threshold of visible injury. Short exposure periods to gas in fumigation chambers are not expected to promote visible injury or gross reductions in growth but may alter stomatal resistance, transpiration, or photosynthesis. Absorption and adsorption rates may be calculated and gas concentrations for definitive testing selected based on the calculated sorption rates.

(iii) Alternatively, the seed germination/root elongation test or other appropriate phytotoxicity test may be used to establish the appropriate upper concentration for testing.

(4) *Definitive test.* (i) The purpose of the uptake and translocation test is to determine the propensity for a chemical's accumulation in plants or plant parts.

(ii) At least 3 concentrations of chemical, exclusive of controls, should be used in the uptake test. Recommended concentrations would be a descending geometric progression from the upper concentration tested (i.e. 100, 50, 25 mg/l). A minimum of 6 replicate pots per concentration, each containing from one to four seedlings, should be used. If techniques other than radioistopes are used to determine uptake, more replicates may be required to provide sufficient plant materials for analysis. Test chemicals should be added to the hydroponic or nutrient solution or coated on glass beads for the root uptake test; or sprayed, dusted, or gassed directly on the foliage in the foliage uptake tests. Only untreated seed (not treated with fungicides, repellants, etc.) taken from the same lot, and year or season of collection should be used in a given test.

(iii) Control pots should be included in the experimental design and should be used in each run. In addition, a carrier control should be used for those chemicals that need to be solubilized.

(iv) If plants are to be grown hydroponically, seeds should be planted in plugs of cotton or glass-wool supported in the tops of the containers. When sand or glass beads are used, the recommended planting procedure is to fill potting containers with sand or glass beads to within 2.5 cm of the top and to sow seeds directly. After germination, the seedlings should be thinned by pinching the stem at the support surface. From one to four seedlings per potting container are required depending on species tested, the size of the containers, and the size to which the plants will grow. When plants are grown hydroponically, one plant per pot will probably be the preferred method. The number of plants selected should provide sufficient biomass for analytical procedures. A greater number of plants may be required depending on species tested, duration of test, and analytical procedures. Too many plants in a container may actually reduce the growth and biomass.

(v) Alternate planting methods may be required when the chemical is highly volatile. An impervious barrier of polyethylene film, a modification of the double pot method, a glass plate, or other appropriate apparatus should be used to prevent volatilization from the root zone. Seeds should be germinated in the dark at 25°C and seedlings with radicle length in the median range transplanted into the potting containers. The seedlings should be positioned such that their roots are exposed to the support media while the shoots pass through holes in the barrier. A ring of inert, non-phytotoxic, pliable putty should be used to seal the holes around the stems. Control pots should be handled identically except there is no exposure to the test chemical. This transplanting procedure, without the volatilization barrier, is also recommended when the test chemical is adsorbed to the support medium.

(vi) Hydroponic solutions should be aerated and sand or glass filled potting containers should be periodically filled with nutrient solution and drained to provide aeration. For root exposure tests, the test chemical should be added to the nutrient solution or directly to substrate. The entire test solution should be replaced weekly, or earlier if the concentration of chemical in the test or nutrient solution varies by more than 20 percent of that specified. The volume of solution added should be recorded.

(vii) The test consists of one run for each of two specified plant species. The duration of a run, for solid and liquid chemicals, should be equal to the length of time required for the particular test variety to achieve sufficient biomass for testing. The duration of a run for gaseous chemicals should be the length of time required to make the specified gas exchange measurements. For a particular chemical, a run is defined as exposure of the plant species to three concentrations of test chemical with a minimum of six replicate pots and appropriate controls. Exposure is followed by extraction and analysis for parent compound, metabolites, and bound residues in plant tissues, and in the whole plants for solids, liquids, and gasses or by calculating rates of absorption and adsorption of gasses.

(viii) Visible effects (stunting of growth, discloration, chlorosis and/or necrosis of the leaves, decreased moisture content, or morphological abnormalities, etc.) should be recorded.

(ix) A randomized complete block design is recommended for this test, with blocks delineated within the chambers or over greenhouse benches and randomization of treatments occurring within the blocks. If, because of very large pots and plants, there exists inadequate space within chambers for blocking, total randomization within chambers is acceptable. This design is also appropriate for the growth of plants to be used for foliar exposure with gas.

(x) Irradiation measurements should be taken at the top of the plant canopy and the mean, plus a maximum and a minimum value, determined over the plant-growing area. These measurements should be taken at the start of the test, at biweekly intervals during the test, and at test termination. If the test is conducted in a greenhouse facility, hourly measurements of irradiation should be recorded and presented as daily total irradiance plus representative hourly curves for clear sky conditions and cloudy days.

(xi) Temperature and humidity measurements should be measured daily at the top of the plant canopy during each light and dark period.

(xii) Measurements of carbon dioxide concentration should be made at the top of the plant canopy (of chamber-growth plants) on a "continuous" basis.

(xiii) The amount of water and nutrient solution depleted each week should be recorded, to observe changes in evapotranspiration rates which may indicate stress. Furthermore, these data will be used to compute chemical uptake per volume of water transpired for the uptake test.

(5) [Reserved]

(6) *Analytical measurements*—(i) *Solid or liquid test chemicals.* (A) Stock solutions should be diluted with glass distilled or deionized water to obtain the test solutions. Standard analytical methods, if available, should be used to establish concentrations of these solutions and should be validated before beginning the test. An analytical method is not acceptable if likely degradation products of the chemical, such as hydrolysis and oxidation products, give positive or negative interference. The pH of these solutions should also be measured prior to use.

(B) The entire plant should be harvested, rinsed with a minimum amount of water (which is returned to the nutrient solution), and separated into its respective organs as follows: carrot—root peels, peeled roots, and tops; cucumber—fruit, vines plus leaves, and roots; corn—kernels, husk plus cob, stalk plus leaves, and roots; lettuce—tops and roots; onion—bulb and tops; ryegrass—tops and roots; soybean—grain, chaff plus tops, and roots; oats—grain, chaff plus tops, and roots; tomato—fruit, vines, and roots; cabbage—head and roots. Plants from two pots in each treatment may be pooled, giving 3 replicate sample pools per treatment. After the fresh weights of the plant organs are obtained, each pool of organs should be subsampled for percent moisture determinations by drying, at 70 °C for 24 hours in a forced-air drying oven, and weighing. Percent moisture determined from these subsamples is used to correct for dry weight of the fresh samples which should then be homogenized and extracted in organic and aqueous solvents. If radioisotopes are used, the amount of test chemical in each extract should be determined by liquid or solid scintillation depending on the type of radiation; otherwise, the amount of chemical should be determined by standard methods. At test completion, the root support material should be washed in organic and then aqueous solvent and analyzed for test chemical before discarding.

(C) A suggested extraction procedure appropriate for many organic chemicals is as follows: One gram of plant material should be homogenized with one gram of solvent-washed anhydrous sodium sulfate in 4 ml of hexane or acetonitrile. The homogenate should be filtered or centrifuged, the solid residue rinsed with an appropriate organic solvent, and the filtrate or supernatant combined with the rinse. The solid residue should be extracted by sequentially (*1*) homogenizing in water, (*2*) centrifuging and decanting the supernatant, (*3*) extracting of the pellet with 6N hydrochloric acid at 60°C for 10 hours, (*4*) subsequently digesting with 10N potassium hydroxide, and (*5*) combining supernatants. The resulting solution should be analyzed by liquid scintillation spectrometry or GLC methodology. The organic extract should be evaporated under vacuum to a sufficiently small volume for thin layer chromatography (TLC) and co-chromatographed on silica gel plates with known standards of the parent chemical. If radioisotopes were used, the chromatographs could be scanned for radioactive substances on a radiochromatogram scanner. Alternatively, zones may be removed from the plates, extracted, and the quantity of chemical from each zone determined by liquid scintillation spectrometry or GLC methodology. The unextractable chemical in the remaining residue may be determined by oxidizing the residue in a complete combustion oxidizer.

(ii) *Gaseous test chemicals.* (A) A gas exposure system yields requisite data for a direct calculation of uptake rates. At steady state, chemical uptake may be determined by a mass balance calculation. Correction for adsorption to surfaces of the exposure chamber should be made by operating the system without plants. Pots filled with hydroponic solution or support media should be included in the system adsorption calibration. Consequently, chemical analyses of plant tissues exposed to gaseous chemicals may not be required in order to demonstrate and quantitate uptake rates.

(B) Altered rates of net photosynthesis, transpiration, and stomatal conductance are anticipated as a result of chemical uptake. Rates of these physiological processes before, during, and after exposure to the gaseous chemical should be determined. Data required for these calculations are available as a consequence of maintaining the specified environmental conditions within the fumigation chamber.

(iii) *Numerical.* Mass of pooled plant organs and pooled whole plants should be measured for the uptake and translocation test and subjected to chemical analyses (above) to quantify free parent test chemical, its metabolites and soluble and bound residues. Mass balance of the test chemical and evapotranspiration rates of the plants are also determined. Means and standard deviations should be calculated and plotted for each of the above for every treatment and control. The data should also be subjected to an analysis of variance.

(d) *Test conditions*—(1) *Test species.* (i) Test plants recommended for the uptake test include:

(A) *Lycopersicon esculentum* (tomato).

(B) *Cucumis sativus* (cucumber).

(C) *Lactuca sativa* (lettuce).

(D) *Glycine max* (soybean).

(E) *Brassica oleracea* (cabbage).

(F) *Avena sativa* (oat).

(G) *Lolium perenne* (perennial ryegrass).

(H) *Allium cepa* (common onion).

(I) *Daucus carota* (carrot).

(J) *Zea mays* (corn).

(ii) Other species of economic or ecologic importance to the region of impact, may also be appropriate and selected for testing. Two species of potentially differing sensitivity should be selected such as a monocotyledonous and a dicotyledonous species. It is further suggested that the test plants selected should be of different growth forms, e.g., a root crop and a leaf crop.

(2) *Facilities*—(i) *Apparatus.* Greenhouses, environmental chambers, or growth rooms should provide adequate environmental control to meet the carbon dioxide, humidity, irradiation, photoperiod, and temperature specifications. Chambers should be designed to prevent escape of internal air into the external environment other than through appropriate filtering material or media to prevent contamination of the external environment with radioactive and/or test substances. Laboratory facilities for plant extractions and chemical determinations should include nonporous floor covering, absorbent bench covering with nonporous backing, and adequate disposal facilities to accommodate plant nutrient, test, and wash solutions containing radioisotope and/or test chemical at the end of each run, and any bench covering, lab clothing, or other contaminated materials.

(ii) *Containers and support media.* For testing purposes, at least 24 polyethylene pots sufficiently large to grow at least 5 plants up to 28 days or one to three plants to maturity are required. If plants are grown hydroponically, one plant per pot may be the preferred method. If a carrier control is needed, 30 pots are used. Potting containers used in each experiment should be of equal size and volume and possess the same configuration. When sand or glass beads are used the potting containers should be filled to within 2.5 cm of their tops with sand or glass beads. Perlite, vermiculite, native soils, etc., should not be used for root support. Potting containers should be covered with opaque polyethylene bags to exclude light and minimize volatilization of test chemical.

(iii) *Cleaning and sterilization.* Potting containers, nutrient storage containers, and root support medium should be cleaned before use. All equipment should be washed according to good standard laboratory procedures to remove any residues remaining from manufacturing or use. A dichromate solution should not be used for cleaning beads or pots. Rooting media other than glass beads should be discarded at the end of the experiment. Disposal should conform to existing regulations.

(iv) *Nutrient media.* Half-strength modified Hoagland nutrient solution should be utilized as nutrient media for this test. Hydroponic solution should be aerated and sand or glass beads potting containers should be filled with nutrient solution and drained periodically. An automated system design is recommended.

(3) *Test parameters.* Environmental conditions should be maintained as specified below:

(i) Carbon dioxide concentrations at 350 ± 50 ppm.

(ii) Relative humidity approaching 70 ± 5 percent during light periods and 90 percent during dark periods.

(iii) Irradiation, measured at 1 meter from the source, at 350 ± 50 $\mu E/m^2$ sec at 400 to 700 nm.

(iv) Photoperiod of 16 hours light and 8 hours darkness for all species except soybean which should be provided with 11 hours light and 13 hours darkness prior to flowering.

(v) Day/night temperatures at 25°/20° ± 3°C.

(e) *Reporting.* Reporting requirements of Part 792—Good Laboratory Practice Standards of this chapter apply to this guideline. Concentrations should be expressed in appropriate weight units per grams of dry plant material and of water lost by evapotranspiration. Data should also include initial and final total concentration of the test chemical in the growth media. These data will be used to compute mass balance. The following should be reported for each of the species tested in tabular form:

(1) *Solid and liquid test chemicals.* (i) Concentration of chemical in nutrient solution and root support material when chemical is soluble in water or solubilized with a carrier compound, as well as the concentration of carrier compound in nutrient solution when carrier is used, or the quantity of chemical per unit weight of root support material when it is coated on the material.

(ii) The quantity of chemical, the concentration at which it was applied, and the number of applications for those chemicals applied to the foliage.

(iii) Environmental conditions (day/night temperatures, relative humidity, light intensity, carbon dioxide concentration, and photoperiod) and the occurrence and extent of any disruption of environmental control facilities.

(iv) Mass of each pool of plant organs and by summation, the mass of whole plants (dry weight after 24 hours at 70 °C).

(v) Concentration of free parent test chemical, metabolites and soluble residues, and bound residues in pooled plant organs and pooled whole plants.

(vi) Mass balance of chemical.

(vii) Mean evapotranspiration rate per plant.

(viii) Visible effects of chemical, if any, on the intact plants.

(ix) Analysis of variance, F-test, means, and standard deviation about the mean are calculated under paragraphs (e)(1) (iv), (v), (vi), and (vii) of this section.

(2) *Gaseous test chemicals.* (i) Concentration of gaseous test chemical at inflow and outflow ports.

(ii) Environmental conditions within gas exposure system (air temperature, dew point temperature or water vapor pressure of incoming and outgoing air streams, light intensity, air speed within chamber, carbon dioxide concentration at inflow and outflow ports, gas flow rate into and out of exposure system).

(iii) Mass (dry weight after 24 hours at 70 °C) of leaves and stems and surface area (one side of leaves) in the exposure system.

(iv) Calculated measurements of photosynthesis, transpiration, and stomatal conductance before, during, and after exposure to test chemicals.

(v) Visible effects of chemical, if any, on the plants.

(vi) Analysis of variance, F-test, means, and standard deviation about the mean are calculated for each of the following:

(A) Steady state rates of photosynthesis, transpiration, and chemical uptake before, during, and after fumigation.

(B) Stomatal conductance or leaf diffusion resistance before, during, and after fumigation.

(vii) If uptake is determined by direct chemical analysis of plant tissues, then the reporting requirements also include:

(A) Concentration of free parent test chemical, metabolites and soluble residues, and bound residues in pooled plant organs and pooled whole plants.

(B) Mass balance of the chemical.

(C) Analysis of variance, F-test, means and standard deviation about the mean under paragraphs (e)(2)(vi) (A) and (B) of this section.

# PART 798—HEALTH EFFECTS TESTING GUIDELINES

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### Subpart H—Special Studies

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AUTHORITY: 15 U.S.C. 2603.

SOURCE: 50 FR 39397, Sept. 27, 1985, unless otherwise noted.

## Subpart A—[Reserved]

## Subpart B—General Toxicity Testing

### § 798.1100 Acute dermal toxicity.

(a) *Purpose.* In the assessment and evaluation of the toxic characteristics of a substance, determination of acute dermal toxicity is usually an initial step. It provides information on health hazards likely to arise from short-term exposure by the dermal route. Data from an acute study may serve as a basis for classification and labeling. It is traditionally a step in establishing a dosage regimen in subchronic and other studies and may provide initial information on dermal absorption and the mode of toxic action of a substance. An evaluation of acute toxicity data should include the relationship, if any, between the animals' exposure to the test substance and the incidence and severity of all abnormalities, including behavioral and clinical abnormalities, the reversibility of observed abnormalities, gross lesions, body weight changes, effects on mortality, and any other toxic effects.

(b) *Definitions.* (1) Acute dermal toxicity is the adverse effects occurring within a short time of dermal application of a single dose of a substance or multiple doses given within 24 hours.

(2) Dose is the amount of test substance applied. Dose is expressed as weight of test substance (g, mg) per unit weight of test animal (e.g., mg/kg).

(3) Dose-response is the relationship between the dose and the proportion of a population sample showing a defined effect.

(c) *Approaches to the determination of acute toxicity.* (1) At present, the evaluation of chemicals for acute toxicity is necessary for the protection of public health and the environment. When animal testing is required for this purpose, this testing should be done in ways that minimize numbers of animals used and that take full account of their welfare.

(2) EPA recommends the following means to reduce the number of animals used to evaluate acute effects of chemical exposure while preserving its ability to make reasonable judgments about safety:

(i) Attempt the use of existing data on structurally related chemicals.

(ii) If data for calculating an $LD_{50}$ are needed, perform an acute toxicity study whereby the value of the data derived from the investment of animal lives is enhanced. EPA does not encourage the use of animals solely for the calculation of an $LD_{50}$.

(iii) Use methods that minimize the numbers of animals in the test.

(3) The following provides an expanded discussion of these principles and their application to the evaluation of acute toxicity of chemicals.

(i) *Using data from structurally related chemicals.* In order to minimize the need for animal testing, the Agency encourages the review of existing acute toxicity information on chemical substances that are structurally related to the agent under investigation. In certain cases, one may be able to glean enough information from these surrogate chemicals to make preliminary safety evaluations that may obviate the need for further animal testing.

(ii) *"Limit" test.* When acute lethality data are desirable, EPA's test guideline encourages the use of methods that minimize the requirement for animals, sometimes by a factor of 90 percent as compared to the more traditional $LD_{50}$ test. In the "limit" test, a single group of animals receives a large dose (2 g/kg body weight) of the agent by the dermal route. If no lethality is demonstrated, no further testing for acute dermal toxicity is pursued.

(iii) *Estimation of lethal dose.* For those substances demonstrating lethality in a "limit" test or for substances for which there are data on structurally related chemicals that indicate potential acute toxicity below 2 g/kg, the Agency can use estimates of the dose associated with some level of acute lethality that are derived from a study comprising three doses as described in this guideline. With such an approach, use of greater numbers of animals or increased numbers of dose levels are not necessary.

(iv) *Multiple endpoint evaluation.* The Agency stresses the simultaneous monitoring of several endpoints of toxicity in animals in a single acute study including sublethal effects as well as lethality. Dosed animals are observed for abnormal behavioral manifestations such as increased salivation or muscular incoordination, in addition to the recovery from these effects during the observation period. Both dead and surviving animals are autopsied to evaluate gross anatomical evidence of organ toxicity. In selected cases, additional testing may be justified to characterize better the kinds of abnormalities that have been found in the organs of the autopsied animals.

(4) These sound, scientific practices represent some of the means which maximize the utility of the data obtained from a limited number of test animals to achieve a balance between protecting humans and the environment, and the welfare and utilization of laboratory animals. When animal testing is, nonetheless, determined to be necessary to achieve this balance, the following test method incorporates the principles discussed in this section.

(d) *Principle of the test method.* When conducting acute toxicity testing, exposure by dermal application is recommended for chemicals where exposure of humans by the dermal route is likely. A single exposure and a 14-day observation period are used. The test substance is applied dermally in graduated doses to several groups of experimental animals, one dose being used per group. For the limit test, however, only one group is tested at a single (high) dose. Subsequent to exposure, systematic daily observations of effects and deaths are made. Based on the results of cage-side observations or gross necropsy, the tester may decide to initiate histopathological review of certain organs, and/or additional clinical laboratory tests. Animals that die during the test are necropsied, and at the conclusion of the observation period, the surviving animals are sacrificed and are necropsied.

(e) *Limit test.* If a test at a dose level of at least 2 g/kg body weight produces no compound-related mortality, then a study using three dose levels will not be necessary.

(f) *Test procedures*—(1) *Animal selection*—(i) *Species and strain.* The rat, rabbit, or guinea pig may be used. The albino rabbit is preferred because of its size, skin permeability, and extensive data base. Commonly used laboratory strains should be employed. If a species other than the three indicated above is used, the tester should provide justification and reasoning for its selection.

(ii) *Age.* Young adult animals should be used. The following weight ranges are suggested to provide animals of a size which facilitates the conduct of the test: rats, 200 to 300 g; rabbits 2.0 to 3.0 kg; guinea pigs 350 to 450 g.

(iii) *Sex.* (A) Equal numbers of animals of each sex with health intact skin should be used for each dose level.

(B) The females should be nulliparous and nonpregnant.

(iv) *Numbers per dose group.* At least 5 animals of each sex should be used at each dose level.

(2) *Control groups.* A concurrent untreated control is not necessary. A vehicle control group should be run concurrently except when historical data are available to determine the acute toxicity of the vehicle.

(3) *Dose levels and dose selection.* (i) Three dose levels should be used and spaced appropriately to produce test groups with a range of toxic effects and mortality rates. The data should be sufficient to produce a dose-response curve and permit an acceptable estimation of the median lethal dose. Range finding studies using single animals may help to estimate the positioning of the dose groups so that no more than three dose levels will be necessary.

(ii) *Vehicle.* (A) Where necessary, the test substance is dissolved or suspended in a suitable vehicle. It is recommended that wherever possible the use of an aqueous solution be considered first, followed by consideration of a solution in oil (e.g., corn oil) and then by possible solution in other vehicles. For non-aqueous vehicles the toxic characteristics of the vehicle should be known, and if not known should be determined before the test.

(B) When testing solids, which may be pulverized if appropriate, the test substance should be moistened sufficiently with water or, where necessary, a suitable vehicle to ensure good contact with skin. When a vehicle is used, the influence of the vehicle on penetration of skin by the test substance should be taken into account.

(4) *Exposure duration.* The test substance should be administered over a period not exceeding 24 hours.

(5) *Observation period.* The observation period should be at least 14 days. Although 14 days is recommended as a minimum period, the duration of observation should not be fixed rigidly. It should be determined by the toxic reactions, rate of onset and length of recovery period, and may thus be extended when considered necessary. The time at which signs of toxicity appear and disappear, their duration and the time to death are important, especially if there is a tendency for deaths to be delayed.

(6) *Preparation of animal skin.* (i) Shortly before testing, fur should be clipped from the dorsal area of the trunk of the test animals. Shaving may be employed, but is should be carried out approximately 24 hours before the test. Care must be taken to avoid abrading the skin, which would alter its permeability.

(ii) Not less than 10 percent of the body surface area should be clear for the application of the test substance. The weight of the animal should be taken into account when deciding on the area to be cleared and on the dimensions of any covering used.

(7) *Application of test substance.* (i) The test substance should be applied uniformly over an area which is approximately 10 percent of the total body surface area. With highly toxic substances the surface area covered may be less, but as much of the area should be covered with as thin and uniform a film as possible.

(ii) The test substance should be held in contact with the skin with a porous gauze dressing and nonirritating tape throughout a 24-hour exposure period. The test site should be further covered in a suitable manner to retain the gauze dressing and test substance and ensure that the animals cannot ingest the test substance. Restrainers may be used to prevent the ingestion of the test substance, but complete immobilization is not a recommended method.

(iii) At the end of the exposure period, residual test substance should be removed where practicable using water or an appropriate solvent.

(8) *Observation of animals.* (i) A careful clinical examination should be made at least once each day.

(ii) Additional observations should be made daily, especially in the early days of the study. Appropriate actions should be taken to minimize loss of animals to the study (e.g., necropsy or refrigeration of those animals found dead and isolation of weak or moribund animals).

(iii) Cage-side observations should include, at the least, evaluations of the skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behavior pattern. Particular attention should be directed to observation of tremors, convulsions, lethargy, other signs of central nervous depression, salivation and diarrhea.

(iv) Individual weights of animals should be determined shortly before the test substance is applied, weekly thereafter, and at death. Changes in weights should be calculated and recorded when survival exceeds one day.

(v) The time of death should be recorded as precisely as possible.

(vi) At the end of the test, surviving animals should be weighed and sacrificed.

(9) *Gross pathology.* A gross necropsy should be performed on all animals under test. All gross pathology changes should be recorded.

(10) *Additional evaluations.* In animals surviving 24 hours or more, clinical chemistry tests or microscopic examination of organs showing evidence of gross pathology should be considered because they may yield additional useful information on the induced toxic effects.

(g) *Data and reporting*— (1) *Treatment of results.* Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, body weights, time of death of individual animals at different dose levels, number of animals displaying other signs of toxicity, description of toxic effects and necropsy findings.

(2) *Evaluation of results.* An evaluation of results should include the relationship, if any, between the dose of the test substance and the incidence, severity and reversibility of all abnormalities, including behavioral and clinical effects, gross lesions, body weight changes, effects on mortality, and any other toxicological effects.

(3) *Test report.* In addition to the reporting requirements as specified under 40 CFR Part 792, Subpart J, the following specific information should be reported:

(i) Tabulation of response data by sex and dose level (i.e., number of animals exposed; number of animals showing signs of toxicity; number of animals dying).

(ii) Dose-response curves for mortality and other toxic effects (when permitted by the method of determination).

(iii) Description of toxic effects including their time of onset, duration, reversibility, and relationship to dose.

(iv) Time of death after dosing.

(v) Body weight data.

(vi) Gross pathology findings.

(vii) Histopathology findings and any additional clinical chemistry evaluations, if performed.

(h) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Bliss, C.I. "The determination of the dosage mortality curve from small numbers," *Quarterly Journal of Pharmacy and Pharmacology.* 11:192-216 (1938).

(2) Finney, D.G. "Chapter 3—Estimation of the median effective dose, Chapter 4—Maximum likelihood estimation," *Probit Analysis.* 3rd Ed. (London: Cambridge University Press 1971).

(3) Litchfield, J.T., Jr., Wilcoxon, F. "A simplified method of evaluating

dose-effect experiments," *Journal of Pharmacology and Experimental Therapeutics.* 96:99-115 (1949).

(4) Miller, L.C., Tainter, M.L. "Estimation of the $ED_{50}$ and its error by means of logarithmic graph paper," *Proceedings of the Society for Experimental Biology and Medicine.* 57:261-264 (1944).

(5) National Academy of Sciences. "Principles and procedures for evaluating the toxicity of household substances," A report prepard by the Committee for the Revision of NAS Publication 1138, under the auspices of the Committee on Toxicology, National Research Council, National Academy of Sciences, Washington, DC (1977).

(6) Thompson, W.R. "Use of moving averages and interpolation to estimate median effective dose," *Bacteriological Review.* 11:115-145 (1947).

(7) Weil, C.S. "Tables for convenient calculation of median effective dose and instructions in their use," *Biometrics.* 8:249-263 (1952).

(8) World Health Organization. "Part I. Environmental Health Criteria 6," *Principles and Methods for Evaluating the Toxicity of Chemicals.* Geneva: World Health Organization (1978).

## § 798.1150 Acute inhalation toxicity.

(a) *Purpose.* In the assessment and evaluation of the toxic characteristics of a substance that may be inhaled, determination of acute toxicity is usually an initial step. It provides information on health hazards likely to arise from short-term exposure by the inhalation route. Data from an acute study may serve as a basis for classification and labeling. It is traditionally a step in establishing a dosage regimen in subchronic and other studies and may provide initial information on the mode of toxic action of a substance. An evaluation of acute toxicity data should include the relationship, if any, between the animals' exposure to the test substance and the incidence and severity of all abnormalities, including behavioral and clinical abnormalities, the reversibility of observed abnormalities, gross lesions, body weight changes, effects on mortality, and any other toxic effects.

(b) *Definitions.* (1) Acute inhalation toxicity is the adverse effects caused by a substance following a single uninterrupted exposure by inhalation over a short period of time (24 hours or less) to a substance capable of being inhaled.

(2) Aerodynamic diameter applies to the size of particles of aerosols. It is the diameter of a sphere of unit density which behaves aerodynamically as the particle of the test substance. It is used to compare particles of different size and densities and to predict where in the respiratory tract such particles may be deposited. This term is used in contrast to measured or geometric diameter which is representative of actual diameters which in themselves cannot be related to deposition within the respiratory tract.

(3) The geometric mean diameter or the median diameter is the calculated aerodynamic diameter which divides the particles of an aerosol in half based on the weight of the particles. Fifty percent of the particles by weight will be larger than the median diameter and 50 percent of the particles will be smaller than the median diameter. The median diameter and its geometric standard deviation is used to statistically describe the particle size distribution of any aerosol based on the weight and size of the particles.

(4) Inhalable diameter refers to that aerodynamic diameter of a particle which is considered to be inhalable for the organism. It is used to refer to particles which are capable of being inhaled and may be deposited anywhere within the respiratory tract from the trachea to the alveoli. For man, the inhalable diameter is considered as 15 micrometers or less.

(5) Dose response is the relationship between the dose (or concentration) and the proportion of a population sample showing a defined effect.

(c) *Approaches to the determination of acute toxicity.* (1) At present, the evaluation of chemicals for acute toxicity is necessary for the protection of public health and the environment. When animal testing is required for this purpose, this testing should be done in ways that minimize numbers of animals used and that take full account of their welfare.

(2) EPA recommends the following means to reduce the number of animals used to evaluate acute effects of chemicals exposure while preserving its ability to make reasonable judgments about safety:

(i) Attempt the use of existing data on structurally related chemicals.

(ii) If data for calculating an $LC_{50}$ are needed, perform an acute toxicity study whereby the value of the data derived from the investment of animal lives is enhanced. EPA does not encourage the use of animals solely for the calculation of an $LC_{50}$.

(iii) Use methods that minimize the numbers of animals in the test.

(3) The following provides an expanded discussion of these principles and their application to the evaluation of acute toxicity of chemicals.

(i) *Using data from structurally related chemicals.* In order to minimize the need for animal testing, the Agency encourages the review of existing acute toxicity information on chemical substances that are structually related to the agent under investigation. In certain cases one may be able to glean enough information from these surrogate chemicals to make preliminary safety evaluations that may obviate the need for further animal testing.

(ii) *"Limit" test.* If a test at an exposure of 5 mg/l (actual concentration of respirable substances) for 4 hours or, where this is not possible due to physical or chemical properties of the test substance, the maximum attainable concentration, using the procedures described for this study, produces no compound-related mortality, then a full study using three dose levels will not be necessary.

(iii) *Estimation of lethal dose.* For those substances demonstrating lethality in a "limit" test or for substances for which there are data on structurally related chemicals that indicate potential acute toxicity below 5 mg/l, the Agency can use estimates of the dose associated with some level of acute lethality that are derived from a study comprising three doses as described in this guideline. With such an approach, use of greater numbers of animals or increased numbers of dose levels are not necessary.

(iv) *Multiple endpoint evaluation.* The Agency stresses the simultaneous monitoring of several endpoints of toxicity in animals in a single acute study including sublethal effects as well as lethality. Dosed animals are observed for abnormal behavioral manifestations such as increased salivation or muscular incoordination, in addition to the recovery from these effects during the observation period. Both dead and surviving animals are autopsied to evaluate gross anatomical evidence of organ toxicity. In selected cases, additional testing may be justified to characterize better the kinds of abnormalities that have been found in the organs of the autopsied animals.

(4) These sound, scientific practices represent some of the means which maximize the utility of the data obtained from a limited number of test animals to achieve a balance between protecting humans and the environment, and the welfare and utilization of laboratory animals. When animal testing is, nonetheless, determined to be necessary to achieve this balance, the following test method incorporates the principles discussed in this section.

(d) *Principle of the test method.* When conducting acute toxicity testing, exposure by inhalation is recommended for chemicals where exposure of humans by inhalation is likely. A single exposure and a 14-day observation period are used. The test substance is administered in graduated doses to several groups of experimental animals, one dose being used per group. For the limit test, however, only one group is tested at a single (high) dose. Subsequent to exposure, systematic daily observations of effects and deaths are made. Based on the results of cage-side observations or gross necropsy, the tester may decide to initiate histopathological review of certain organs, and/or additional clinical laboratory tests. Animals that die during the test are necropsied, and at the conclusion of the observation period, the surviving animals are sacrificed and are necropsied.

(e) *Limit test.* If a test at a dose level of at least 5 mg/l (actual concentration of respirable substances) for 4 hours or, where this is not possible due to physical or chemical properties of the test substance, the maximum attainable concentration, produces no compound-related mortality, then a full study using three dose levels will not be necessary.

(f) *Test procedures*—(1) *Animal selection*—(i) *Species and strain.* Although several mammalian test species may be used, the rat is the preferred species. Commonly used laboratory strains should be employed. If another mammalian species is employed, the tester should provide justification and reasoning for its selection.

(ii) *Age.* Young adult animals should be used. The weight variation of animals used in a test should not exceed ± 20 percent of the mean weight for each sex.

(iii) *Sex.* (A) Equal numbers of animals of each sex should be used for each dose level.

(B) The females should be nulliparous and nonpregnant.

(iv) *Numbers per dose group.* At least five animals of each sex should be used at each dose level.

(2) *Control groups.* A concurrent untreated control is not necessary. Where a vehicle is used to help generate an appropriate concentration of the substance in the atmosphere, a vehicle control group should be used when historical data are not available or adequate to determine the acute toxicity of the vehicle.

(3) *Dose levels and dose selection.* (i) Three exposure concentrations should be used and spaced appropriately to produce test groups with a range of toxic effects and mortality rates. The data should be sufficient to produce a dose-response curve and permit an acceptable estimation of the median lethal concentration. Range-finding studies using single animals may help to estimate the positioning of the test groups so that no more than three doses will be necessary.

(ii) Where necessary, a suitable vehicle may be added to the test substance to help generate an appropriate concentration of the test substance in the atmosphere. If a vehicle or diluent is needed, ideally it should not elicit important toxic effects itself or substantially alter the chemical or toxicological properties of the test substance.

(iii) In the case of potentially explosive test substances, care should be taken to avoid generating explosive concentrations.

(iv) To establish suitable exposure concentrations, a trial test is recommended.

(4) *Exposure duration.* The duration of exposure should be at least 4 hours after equilibration of the chamber concentrations.

(5) *Observation period.* The observation period should be at least 14 days. Although 14 days is recommended as a minimum period, the duration of observation should not be fixed rigidly. It should be determined by the toxic reactions, rate of onset and length of recovery period, and may thus be extended when considered necessary. The time at which signs of toxicity appear and disappear, their duration and the time of death are important, especially if there is a tendency for deaths to be delayed.

(6) *Inhalation exposure.* (i) The animals should be tested with inhalation equipment designed to sustain a dynamic air flow of 12 to 15 air changes per hour, ensure an adequate oxygen content of 19 percent and an evenly distributed exposure atmosphere. Where a chamber is used, its design should minimize crowding of the test animals and maximize their exposure to the test substance. This is best accomplished by individual caging. As a general rule to ensure stability of a chamber atmosphere, the total "volume" of the test animals should not exceed 5 percent of the volume of the test chamber. Alternatively, oronasal, head-only, or whole body individual chamber exposure may be used.

(ii) A suitable analytical concentration control system should be used. The rate of air flow should be adjusted to ensure that conditions throughout the equipment are essentially the same. Maintenance of a slight negative pressure inside the chamber will prevent leakage of the test substance into the surrounding area.

(iii) The temperature at which the test is performed should be maintained at 22 °C (± 2°). Ideally, the relative humidity should be maintained between 40 to 60 percent, but in certain instances (e.g., tests on aerosols, use of water vehicle) this may not be practicable.

(7) *Physical measurements.* Measurements or monitoring should be made of the following:

(i) The rate of air flow should be monitored continuously, but should be recorded at least every 30 minutes.

(ii) The actual concentrations of the test substance should be measured in the breathing zone. During the exposure period the actual concentration of the test substance should be held as constant as practicable. Continuous monitoring is desirable. Measurement of actual concentrations should be recorded near the beginning, middle, and end of the exposure period.

(iii) During the development of the generating system, particle size analysis should be performed to establish the stability of aerosol concentrations. During exposure, analysis should be made as often as necessary to determine the consistency of particle size distribution and homogeneity of the exposure stream.

(iv) Temperature and humidity should be monitored continuously but should be recorded at least every 30 minutes.

(8) *Feed and water during exposure period.* Feed should be withheld during exposure. Water may also be withheld in certain cases.

(9) *Observation of animals.* (i) A careful clinical examination should be made at least once each day.

(ii) Additional observations should be made daily, especially in the early days of the study. Appropriate actions should be taken to minimize loss of animals to the study (e.g., necropsy or refrigeration of those animals found dead and isolation of weak or moribund animals).

(iii) Cage-side observations should include, at the least, evaluations of the skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behavior pattern. Particular attention should be directed to observation of tremors, convulsions, lethargy, other signs of central nervous system depression, salivation and diarrhea.

(iv) Individual weights of animals should be determined shortly before the test substance is administered, weekly thereafter, and at death. Changes in weights should be calculated and recorded when survival exceeds one day.

(v) The time of death should be recorded as precisely as possible.

(vi) At the end of the test, surviving animals should be weighed and sacrificed.

(10) *Gross pathology.* A gross necropsy should be performed on all animals under test, with particular reference to any changes in the respiratory tract. Where there are significant signs of toxicity indicating the possible involvement of other organs, these should be examined. All gross pathology changes should be recorded.

(11) *Additional evaluations.* In animals surviving 24 hours or more, clinical chemistry tests or microscopic examination of organs showing evidence of gross pathology should be considered because they may yield additional useful information on the nature of the induced toxic effects.

(g) *Data and reporting*—(1) *Treatment of results.* Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, body weights, time of death of individual animals at different dose levels, number of animals displaying other signs of toxicity, description of toxic effects and necropsy findings.

(2) *Evaluation of results.* An evaluation of results should include the relationship, if any, between the concentration of the test substance and the incidence, severity and reversibility of all abnormalities, including behavioral and clinical effects, gross lesions, body weight changes, effects on mortality, and any other toxicological effects.

(3) *Test report.* In addition to the reporting requirements as specified under 40 CFR Part 792, Subpart J the following specific information should be reported:

(i) *Test conditions.* (A) Description of exposure apparatus including design, type, dimensions, source of air, system for generating particulates and aerosols, method of conditioning air, treatment of exhaust air and the method of housing the animals in a test chamber.

(B) The equipment of measuring temperature, humidity, and particulate aerosol concentrations and size be described.

(ii) *Exposure data.* These should be tabulated and presented with mean values and a measure of variability (e.g., standard deviation) and should include:

(A) Airflow rates through the inhalation equipment.

(B) Temperature and humidity of air.

(C) Nominal concentration (total amount of test substance fed into the inhalation equipment divided by volume of air).

(D) Actual concentration in test breathing zone.

(E) Particle size distribution (e.g., median aerodynamic diameter of particles with standard deviation from the mean).

(iii) *Animal data.* (A) Tabulation of response data by sex and exposure level (i.e., number of animals exposed, number of animals showing signs of toxicity, number of animals dying).

(B) Dose-response curves for mortality and other toxic effects (when permitted by the method of determination).

(C) Description of toxic effects including their time of onset, duration, reversibility, and relationship to dose.

(D) Time of death during or following exposure.

(E) Body weight data.

(F) Gross pathology findings.

(G) Histopathology findings and any additional clinical chemistry evaluation, if performed.

(h) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Bliss, C.I. "The determination of the dosage mortality curve from small numbers," *Quarterly Journal of Pharmacy and Pharmacology.* 11:192-216 (1938).

(2) Finney, D.G. "Chapter 3—Estimation of the median effective dose, Chapter 4—Maximum likelihood estimation," Probit Analysis. 3rd Ed. (London: Cambridge University Press, 1971).

(3) Litchfield, J.T., Jr., Wilcoxon, F. "A simplified method of evaluating dose-effect experiments," *Journal of Pharmacology and Experimental Therapeutics.* 96:99-115 (1949).

(4) Miller, L.C., Tainter, M.L. "Estimation of the $ED_{50}$ and its error by means of logarithmic graph paper," *Proceedings of the Society for Experimental Biology and Medicine.* 57:261-264 (1944).

(5) National Academy of Sciences. "Principles and procedures for evaluating the toxicity of household substances." A report prepared by the Committee for the Revision of NAS Publication 1138, under the auspices of the Committee on Toxicology, National Research Council, National Academy of Sciences, Washington, DC (1977).

(6) Smyth, H.F., Jr., Carpenter, C.P., Weil, C.S., Striegel, J.A. "Range finding toxicity data: List VI," *American Industrial Hygiene Association Journal.* 23:95 (1962).

(7) Thompson, W.R. "Use of moving averages and interpolation to estimate median effective dose," *Bacteriological Review.* 11:115-145 (1947).

(8) Weil, C.S. "Tables for convenient calculation of median effective dose and instructions in their use," *Biometrics.* 8:249-263 (1952).

(9) World Health Organization. "Part I. Environment Health Criteria 6," *Principles and Methods for Evaluating the Toxicity of Chemicals.* Geneva: World Health Organization (1979).

[50 FR 39397, Sept. 27, 1985, as amended at 54 FR 21064, May 16, 1989]

### § 798.1175 Acute oral toxicity.

(a) *Purpose.* In the assessment and evaluation of the toxic characteristics of a substance, determination of acute oral toxicity is usually an initial step. It provides information on health hazards likely to arise from short-term exposure by the oral route. Data from an acute study may serve as a basis for classification and labeling. It is traditionally a step in establishing a dosage regimen in subchronic and other studies and may provide initial information on the mode of toxic action of a substance. An evaluation of acute toxicity data should include the relationship, if any, between the animals' exposure to the test substance and the incidence and severity of all abnormalities, including behavioral and clinical abnormalities, the reversibility of observed abnormalities, gross lesions, body weight changes, effects on mortality, and any other toxic effects.

(b) *Definitions.* (1) Acute oral toxicity is the adverse effects occurring within a short time of oral administration of a single dose of a substance or multiple doses given within 24 hours.

(2) Dose is the amount of test substance administered. Dose is expressed as weight of test substance (g, mg) per unit weight of test animal (e.g. mg/kg).

(3) Dose-response is the relationship between the dose and the proportion of a population sample showing a defined effect.

(c) *Approaches to the determination of acute toxicity.* (1) At present, the evaluation of chemicals for acute toxicity is necessary for the protection of public health and the environment. When animal testing is required for this purpose, this testing should be done in ways that minimize numbers of animals used and that take full account of their welfare.

(2) EPA recommends the following means to reduce the number of animals used to evaluate acute effects of chemical exposure while preserving its ability to make reasonable judgments about safety:

(i) Attempt the use of existing data on structurally related chemicals.

(ii) If data for calculating an $LD_{50}$. are needed, perform an acute toxicity study whereby the value of the data derived from the investment of animal lives is enhanced. EPA does not encourage the use of animals solely for the calculation of an $LD_{50}$.

(iii) Use methods that minimize the numbers of animals in the test.

(3) The following provides an expanded discussion of these principles and their application to the evaluation of acute toxicity of chemicals.

(i) *Using data from structurally related chemicals.* In order to minimize the need for animal testing, the Agency encourages the review of existing acute toxicity information on chemical substances that are structurally related to the agent under investigation. In certain cases one may be able to glean enough information from these surrogate chemicals to make preliminary safety evaluations that may obviate the need for further animal testing.

(ii) *"Limit" test.* When acute lethality data are desirable, EPA's test guideline encourages the use of methods that minimize the requirement for animals, sometimes by a factor of 90 percent as compared to the more traditional $LD_{50}$ test. In the "limit" test, a single group of animals is given a large dose (5 g/kg body weight) of the agent. If no letnality is demonstrated, no further testing for acute oral toxicity is pursued.

(iii) *Estimation of lethal dose.* For those substances demonstrating lethality in a "limit" test or for substances for which there are data on structurally related chemicals that indicate potential acute toxicity below 5 g/kg the Agency can use estimates of the dose associated with some level of acute lethality that are derived from a study comprised of three doses as described in this guideline. With such an approach, use of greater numbers of animals or increased numbers of dose levels are not necessary.

(iv) *Multiple endpoint evaluation.* The Agency stresses the simultaneous monitoring of several endpoints of toxicity in animals in a single acute study including sublethal effects as well as lethality. Dosed animals are observed for abnormal behavioral manifestations such as increased salivation or muscular incoordination, in addition to the recovery from these effects during the observation period. Both dead and surviving animals are autopsied to evaluate gross anatomical evidence of organ toxicity. In selected cases, additional testing may be justified to characterize better the kinds of abnormalities that have been found in the organs of the autopsied animals.

(4) These sound, scientific practices represent some of the means which maximize the utility of the data obtained from a limited number of test animals to achieve a balance between protecting humans and the environment, and the welfare and utilization of laboratory animals. When animal testing is, nonetheless, determined to

be necessary to achieve this balance, the following test method incorporates the principles discussed in this section.

(d) *Principle of the test method.* When conducting acute toxicity testing, exposure by gavage is recommended for chemicals where exposure of humans by the oral route is likely. A single exposure and a 14-day observation period are used. The test substance is administered orally in graduated doses to several groups of experimental animals, one dose being used per group. For the limit test, however, only one group is tested at a single (high) dose. Subsequent to exposure, systematic daily observations of effects and deaths are made. Based on the results of cage-side observations or gross necropsy, the tester may decided to initiate histopathological review of certain organs, and/or additional clinical laboratory tests. Animals that die during the test are necropsied, and at the conclusion of the observation period, the surviving animals are sacrificed and are necropsied.

(e) *Limit test.* If a test at a dose level of at least 5 g/kg body weight produces no compound-related mortality, then a study using three dose levels will not be necessary.

(f) *Test procedures*—(1) *Animal selection*—(i) *Species and strain.* Although several mammalian test species may be used, the rat is the preferred species. Commonly used laboratory strains should be employed. If another species is used, the tester should provide justificaion and reasoning for its selection.

(ii) *Age.* Young adult animals should be used. The weight variation of animals used in a test should not exceed ± 20 percent of the mean weight for each sex.

(iii) *Sex.* (A) Equal numbers of animals of each sex should be used for each dose level.

(B) The females should be nulliparous and nonpregnant.

(iv) *Numbers per dose group.* At least five animals of each sex should be used at each dose level.

(2) *Control groups.* A concurrent untreated control is not necessary. A vehicle control group should be run concurrently except when historical data are available to determine the acute toxicity of the vehicle.

(3) *Dose levels and dose selection.* (i) Three dose levels should be used and spaced appropriately to produce test groups with a range of toxic effects and mortality rates. The data should be sufficient to produce a dose-response curve and permit an acceptable estimation of the median lethal dose. Range-finding studies using single animals may help to estimate the positioning of the dose groups so that no more than three dose levels will be necessary.

(ii) *Vehicle.* Where necessary, the test substance is dissolved or suspended in a suitable vehicle. It is recommended that wherever possible the use of an aqueous solution be considered first, followed by consideration of a solution in oil (e.g., corn oil) and then by possible solution in other vehicles. For nonaqueous vehicles the toxic characteristics of the vehicle should be known, and if not known should be determined before the test.

(iii) *Volume.* The maximum volume of liquid that can be administered at one time depends on the size of the test animal. In rodents, the volume should not exceed 1 ml/100 g body weight, except when an aqueous solution is used where 2 ml/100 g may be administered. Variability in test volume should be minimized by adjusting the concentration to ensure a constant volume at all dose levels.

(4) *Exposure duration.* The test substance should be administered over a period not exceeding 24 hours.

(5) *Observation period.* The observation period should be at least 14 days. Although 14 days is recommended as a mininum period, the duration of observation should not be fixed rigidly. It should be determined by the toxic reactions, rate of onset, and length of recovery period, and may thus be extended when considered necessary. The time at which signs of toxicity appear and disappear, their duration, and the time to death are important, especially if there is a tendency for deaths to be delayed.

(6) *Exposure.* (i) The test substance should be administered in a single dose by gavage, using a stomach tube or suitable intubation cannula.

(ii) Animals should be fasted prior to test substance administration. For the rat, feed should be withheld overnight; for other rodents with higher metabolic rates a shorter period of fasting is appropriate.

(iii) After the substance has been administered, feed may be withheld for an additional 3 to 4 hours.

(iv) If a single dose is not possible, the dose may be given in smaller fractions over a period not exceeding 24 hours. Where a dose is administered in fractions, it may be necessary to provide the animals with food and water depending on the length of the dosing period.

(7) *Observation of animals.* (i) A careful clinical examination should be made at least once each day.

(ii) Additional observations should be made daily, especially in the early days of the study. Appropriate actions should be taken to minimize loss of animals to the study (e.g., necropsy or refrigeration of those animals found dead and isolation of weak or moribund animals).

(iii) Cage-side observations should include, at the least, evaluation of the skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behavior pattern. Particular attention should be directed to observation of tremors, convulsions, lethargy, other signs of central nervous system depression, salivation, and diarrhea.

(iv) Individual weights of animals should be determined shortly before the test substance is administered, weekly thereafter, and at death. Changes in weights should be calculated and recorded when survival exceeds one day.

(v) The time of death should be recorded as precisely as possible.

(vi) At the end of the test, surviving animals should be weighed and sacrificed.

(8) *Gross pathology.* A gross necropsy should be performed on all animals under test. All gross pathology changes should be recorded.

(9) *Additional evaluations.* In animals surviving 24 hours or more, clinical chemistry tests or microscopic examination of organs showing evidence of gross pathology should be considered because they may yield additional useful information on the nature of the induced toxic effects.

(g) *Data and reporting*—(1) *Treatment of results.* Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, body weights, time of death of individual animals at different dose levels, number of animals displaying other signs of toxicity, description of toxic effects and necropsy findings.

(2) *Evaluation of results.* An evaluation of results should include the relationship, if any, between the dose of the test substance and the incidence, severity, and reversibility of all abnormalities, including behavioral and clinical effects, gross lesions, body weight changes, effects on mortality, and any other toxicological effects.

(3) *Test report.* In addition to the reporting requirements as specified under 40 CFR Part 792, Subpart J the following specific information should be reported.

(i) Tabulation of response data by sex and dose level (i.e., number of animals exposed; number of animals showing signs of toxicity; number of animals dying).

(ii) Dose-response curves for mortality and other toxic effects (when permitted by the method of determination).

(iii) Description of toxic effects, including their time of onset, duration, reversibility, and relationship to dose.

(iv) Time of death after dosing.

(v) Body weight data.

(vi) Gross pathology findings.

(vii) Histopathology findings and any additional clinical chemistry evaluations, if performed.

(h) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Balazs, T. "Measurement of acute toxicity," *Methods in Toxicology.* ed. G.E. Paget (Philadelphia: F.A. Davis Co., 1970), pp. 49-82.

(2) Bliss, C.I. "The determination of the dosage mortality curve from small numbers," *Quarterly Journal of Pharmacy and Pharmacology.* 11:192-216 (1938).

(3) Finney, D.G. "Chapter 3—Estimation of the median effective dose,

Chapter 4—Maximum likelihood estimation," *Probit Analysis.* 3rd Edition. (London: Cambridge University Press, 1971).

(4) Hunter, W.J., Lingk, W., Recht, P. "Intercomparison study on the determination of single administration toxicity in rats," *Journal of the Association of Official Analytical Chemists.* 62(4):864-873 (1979).

(5) Litchfield, J.T., Jr., Wilcoxon, F. "A simplified method of evaluating dose-effect experiments," *Journal of Pharmacology and Experimental Therapeutics.* 96:99-115 (1949).

(6) Miller, L.C., Tainter, M.L. "Estimation of the $ED_{50}$ and its error by means of logarithmic graph paper," *Proceedings of the Society for Experimental Biology and Medicine.* 57:261-264 (1944).

(7) National Academy of Sciences. "Principles and procedures for evaluating the toxicity of household substances," a report prepared by the Committee for the Revision of NAS Publication 1138, under the auspices of the Committee on Toxicology, National Research Council, National Academy of Sciences, Washington, DC (1977).

(8) Thompson, W.R. "Use of moving averages and interpolation to estimate median effective dose," *Bacteriological Review.* 11:115-145 (1947).

(9) Weil, C.S. "Tables for convenient calculation of median effective dose and instructions in their use," *Biometrics.* 8:249-263 (1952).

(10) World Health Organization. "Part I. Environmental Health Criteria 6," *Principles and Methods for Evaluating the Toxicity of Chemicals.* Geneva: World Health Organization (1978).

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## Subpart C—Subchronic Exposure

### § 798.2250 Dermal toxicity.

(a) *Purpose.* In the assessment and evaluation of the toxic characteristics of a chemical, the determination of subchronic dermal toxicity may be carried out after initial information on toxicity has been obtained by acute testing. The subchronic dermal study has been designed to permit the determination of the no-observed-effect level and toxic effects associated with continuous or repeated exposure to a test substance for a period of 90 days. The test is not capable of determining those effects that have a long latency period for development (e.g., carcinogenicity and life shortening). It provides information on health hazards likely to arise from repeated exposure by the dermal route over a limited period of time. It will provide information on target organs, the possibilities of accumulation, and can be of use in selecting dose levels for chronic studies and for establishing safety criteria for human exposure.

(b) *Definitions.* (1) Subchronic dermal toxicity is the adverse effects occurring as a result of the repeated daily exposure of experimental animals to a chemical by dermal application for part (approximately 10 percent) of a life span.

(2) Dose in a dermal test is the amount of test substance applied to the skin (applied daily in subchronic tests). Dose is expressed as weight of the substance (g, mg) per unit weight of test animal (e.g., mg/kg).

(3) No-effect level/No-toxic-effect level/No-adverse-effect level/No-observed-effect level is the maximum dose used in a test which produces no observed adverse effects. A no-observed-effect level is expressed in terms of the weight of a test substance given daily per unit weight of test animal (mg/kg).

(4) Cumulative toxicity is the adverse effects of repeated doses occurring as a result of prolonged action on, or increased concentration of the administered test substance or its metabolites in susceptible tissues.

(c) *Principle of the test method.* The test substance is applied daily to the skin in graduated doses to several groups of experimental animals, one dose level per unit group, for a period of 90 days. During the period of application the animals are observed daily to detect signs of toxicity. Animals which die during the test are necropsied, and at the conclusion of the test the surviving animals are sacrificed and necropsied and appropriate histopathological examinations carried out.

(d) *Limit test.* If a test at one dose level of at least 1,000 mg/kg body weight (expected human exposure may indicate the need for a higher dose level), using the procedures described for this study, produces no observable toxic effects and if toxicity would not be expected based upon data of structurally related compounds, then a full study using three dose levels might not be necessary.

(e) *Test procedures*—(1) *Animal selection*—(i) *Species and strain.* A mammalian species shall be used for testing. The rat, rabbit, or guinea pig may be used, although the albino rabbit is preferred. The albino rabbit is preferred because of its size, skin permeability, and extensive data base. Commonly used laboratory strains shall be employed. If another mammalian species is used, the tester shall provide justification/reasoning for its selection.

(ii) *Age.* Young adult animals shall be used. The following weight ranges at the start of the test are suggested in order to provide animals of a size which facilitates the conduct of the test: rats, 200 to 300 g; rabbits, 2.0 to 3.0 kg; guinea pigs, 350 to 450 g.

(iii) *Sex.* (A) Equal numbers of animals of each sex with healthy skin shall be used at each dose level.

(B) The females shall be nulliparous and nonpregnant.

(iv) *Numbers.* (A) At least 20 animals (10 females and 10 males) shall be used at each dose level.

(B) If interim sacrifices are planned, the number shall be increased by the number of animals scheduled to be sacrificed before completion of the study.

(2) *Control groups.* A concurrent control group is required. This group shall be an untreated or sham-treated control group or, if a vehicle is used in administering the test substance, a vehicle control group. If the toxic properties of the vehicle are not known or cannot be made available, both untreated and vehicle control groups are required.

(3) *Satellite group.* A satellite group of 20 animals (10 animals per sex) may be treated with the high dose level for 90 days and observed for reversibility, persistence, or delayed occurrence, of toxic effects for a posttreatment period of appropriate length, normally not less than 28 days.

(4) *Dose level and dose selection.* (i) In subchronic toxicity tests, it is desirable to have a dose-response relationship as well as a no-observed-toxic-effect level. Therefore, at least 3 dose levels with a control and, where appropriate, a vehicle control (corresponding to the concentration of vehicle at the highest exposure level) shall be used. Doses should be spaced appropriately to produce test groups with a range of toxic effects. The data shall be sufficient to produce a dose-response curve.

(ii) The highest dose level should result in toxic effects but not produce severe skin irritation or an incidence of fatalities which would prevent a meaningful evaluation.

(iii) The lowest dose level should not produce any evidence of toxicity. Where there is a usable estimation of human exposure, the lowest dose level should exceed this.

(iv) Ideally, the intermediate dose level(s) should produce minimal observable toxic effects. If more than one intermediate dose is used, the dose levels should be spaced to produce a gradation of toxic effects.

(v) In the low and intermediate groups and in the controls the incidence of fatalities should be low, to permit a meaningful evaluation of the results.

(5) *Exposure conditions.* The animals are treated with test substance, ideally for at least 6 hours per day on a 7-day per week basis, for a period of 90 days. However, based primarily on practical considerations, application on a 5-day per week basis is considered to be acceptable.

(6) *Observation period.* (i) Duration of observation shall be at least 90 days.

(ii) Animals in the satellite group scheduled for followup observations should be kept for at least 28 days further without treatment to detect recovery from, or persistence of, toxic effects.

(7) *Preparation of animal skin.* (i) Shortly before testing, fur shall be clipped from the dorsal area of the trunk of the test animals. Shaving

may be employed, but it should be carried out approximately 24 hours before the test. Repeat clipping or shaving is usually needed at approximately weekly intervals. When clipping or shaving the fur, care should be taken to avoid abrading the skin, which could alter its permeability.

(ii) Not less than 10 percent of the body surface area should be clear for the application of the test substance. The weight of the animal should be taken into account when deciding on the area to be cleared and on the dimensions of any covering used.

(iii) When testing solids, which may be pulverized if appropriate, the test substance should be moistened sufficiently with water or, where necessary, a suitable vehicle to ensure good contact with the skin. When a vehicle is used, the influence of the vehicle on toxicity of and penetration of the skin by the test substance should be taken into account.

(8) *Application of the test substance.* (i) The test substance shall be applied uniformly over an area which is approximately 10 percent of the total body surface area. With highly toxic substances, the surface area covered may be less, but as much of the area shall be covered with as thin and uniform a film as possible.

(ii) During the exposure period, the test substance shall be held in contact with the skin with a porous gauze dressing and nonirritating tape. The test site shall be further covered in a suitable manner to retain the gauze dressing and test substance and ensure that the animals cannot ingest the test substance. Restrainers may be used to prevent the ingestion of the test substance, but complete immobilization is not a recommended method.

(9) *Observation of animals.* (i) Each animal shall be observed daily, and if necessary handled to appraise its physical condition.

(ii) Additional observations shall be made daily with appropriate actions taken to minimize loss of animals to the study (e.g., necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals).

(iii) Signs of toxicity shall be recorded as they are observed, including the time of onset, the degree, and duration.

(iv) Cage-side observations shall include, but not be limited to, changes in skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behavior pattern.

(v) Animals shall be weighed weekly. Feed consumption shall also be determined weekly if abnormal body weight changes are observed.

(vi) At the end of the study period, all survivors in the nonsatellite treatment groups shall be sacrificed. Moribund animals shall be removed and sacrificed when noticed.

(10) *Clinical examinations.* (i) The following examinations shall be made on all animals of each sex in each group:

(A) Certain hematology determinations shall be carried out at least two times during the test period on all groups of animals including concurrent controls: After 30 days of test and just prior to terminal sacrifice at the end of the test period. Hematology determinations which are appropriate to all studies: Hematocrit, hemoglobin concentration, erythrocyte count, total and differential leukocyte count, and a measure of clotting potential such as clotting time, prothrombin time, thromboplastin time, or platelet count.

(B) Certain clinical biochemistry determinations on blood should be carried out at least two times during the test period on all groups of animals including concurrent controls: After 30 days of test and just prior to terminal sacrifice at the end of the test period. Clinical biochemistry test areas which are considered appropriate to all studies: Electrolyte balance, carbohydrate metabolism, and liver and kidney function. The selection of specific tests will be influenced by observations on the mode of action of the substance. Suggested determinations: Calcium, phosphorus, chloride, sodium, potassium, fasting glucose (with period of fasting appropriate to the species), serum glutamic pyruvic transaminase (now known as serum alanine aminotransferase), serum glutamic oxaloacetic transaminase (now known as serum aspartate aminotransferase), ornithine decarboxylase, gamma glutamyl transpeptidase, urea nitrogen, albumen blood creatinine, total bilirubin, and total serum protein measurements. Other determinations which may be necessary for an adequate toxicological evaluation include: Analyses of lipids, hormones, acid/base balance, methemoglobin, and cholinesterase activity. Additional clinical biochemistry may be employed, where necessary, to extend the investigation of observed effects.

(ii) The following examinations shall be made on high dose and control groups. If changes in the eyes are detected all animals should be examined.

(A) Ophthalmological examination, using an ophthalmoscope or equivalent suitable equipment, shall be made prior to exposure to the test substance and at the termination of the study.

(B) Urinalysis is not recommended on a routine basis, but only when there is an indication based on expected or observed toxicity.

(11) *Gross necropsy.* (i) All animals shall be subjected to a full gross necropsy which includes examination of the external surface of the body, all orifices, and the cranial, thoracic, and abdominal cavities and their contents.

(ii) The liver, kidneys, adrenals, brain, and gonads shall be weighed wet, as soon as possible after dissection, to avoid drying. In addition, for the rodent, the brain; for the nonrodent, the thyroid with parathyroids also shall be weighed wet.

(iii) The following organs and tissues, or representative samples thereof, shall be preserved in a suitable medium for possible future histopathological examination: All gross lesions; lungs—which should be removed intact, weighed, and treated with a suitable fixative to ensure that lung structure is maintained (perfusion with the fixative is considered to be an effective procedure); nasopharyngeal tissues; brain—including sections of medulla/pons, cerebellar cortex, and cerebral cortex; pituitary; thyroid/parathyroid; thymus; trachea; heart; sternum with bone marrow; salivary glands; liver; spleen; kidneys; adrenals; pancreas; gonads; uterus; accessory genital organs (epididymis, prostate, and, if present, seminal vesicles); aorta; (skin); gall bladder (if present); esophagus; stomach; duodenum; jejunum; ileum; cecum; colon; rectum; urinary bladder; representative lymph node; (mammary gland); (thigh musculature); peripheral nerve; (eyes); (femur—including articular surface); (spinal cord at three levels—cervical, midthoracic, and lumbar); and (zymbal and exorbital lachrymal glands).

(12) *Histopathology.* The following histopathology shall be performed:

(i) Full histopathology on normal and treated skin and on organs and tissues, listed above, of all animals in the control and high dose groups.

(ii) All gross lesions in all animals.

(iii) Target organs in all animals.

(iv) The tissues listed in parenthesis in paragraph (e)(11)(iii) of this section, if indicated by signs of toxicity or expected target organ involvement.

(v) Lungs of animals (rodents) in the low and intermediate dose groups shall be subjected to histopathological examination for evidence of infection, since this provides a convenient assessment of the state of health of the animals.

(vi) When a satellite group is used, histopathology shall be performed on tissues and organs identified as showing effects in the treated groups.

(f) *Data and reporting*—(1) *Treatment of results.* (i) Data shall be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions, the types of lesions, and the percentage of animals displaying each type of lesion.

(ii) All observed results, quantitative and incidental, should be evaluated by an appropriate statistical method. Any generally accepted statistical method may be used; the statistical methods should be selected during the design of the study.

(2) *Evaluation of results.* The findings of a subchronic dermal toxicity study should be evaluated in conjunction with the findings of preceding studies and considered in terms of the observed toxic effects and the necropsy and histopathological findings. The evaluation should include the relationship between the dose of the test substance and the presence or absence,

the incidence and severity, of abnormalities, including behavioral and clinical abnormalities, gross lesions, identified target organs, body weight changes, effect on mortality and any other general or specific toxic effects. A properly conducted subchronic test should provide a satisfactory estimation of a no-effect level.

(3) *Test report.* In addition to the reporting requirements as specified in the EPA Good Laboratory Practice Standards under 40 CFR Part 792, Subpart J, the following specific information shall be reported.

(i) *Group animal data.* Tabulation of toxic response data by species, strain, sex and exposure level for:

(A) Number of animals dying.

(B) Number of animals showing signs of toxicity.

(C) Number of animals exposed.

(ii) *Individual animal data.* (A) Date of death during the study or whether animals survived to termination.

(B) Date of observation of each abnormal sign and its subsequent course.

(C) Body weight data.

(D) Feed consumption data when collected.

(E) Hematological tests employed and all results.

(F) Clinical biochemistry tests employed and all results.

(G) Necropsy findings.

(H) Detailed description of all histopathological findings.

(I) Statistical treatment of results where appropriate.

(g) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Draize, J.H. "Dermal toxicity," *Appraisal of Chemicals in Food, Drugs and Cosmetics.* The Association of Food and Drug Officials of the United States (1959, 3rd printing 1975). pp. 46-59.

(2) Fitzhugh, O.G. "Subacute toxicity," *Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics.* The Association of Food and Drug Officials of the United States (1959, 3rd printing 1975). pp. 26-35.

(3) National Academy of Sciences. "Principles and Procedures for Evaluating the Toxicity of Household Substances," a report prepared by the Committee for the Revision of NAS Publication 1138, under the auspices of the Committee on Toxicology, National Research Council, National Academy of Sciences, Washington, DC (1977).

(4) World Health Organization. "Part I. Environmental Health Criteria 6,"*Principles and Methods for Evaluating the Toxicity of Chemicals.* (Geneva: World Health Organization, 1978).

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## § 798.2450 Inhalation toxicity.

(a) *Purpose.* In the assessment and evaluation of the toxic characteristics of a gas, volatile substance, or aerosol/particulate, determination of subchronic inhalation toxicity may be carried out after initial information on toxicity has been obtained by acute testing. The subchronic inhalation study has been designed to permit the determination of the no-observed-effect level and toxic effects associated with continuous or repeated exposure to a test substance for a period of 90 days. The test is not capable of determining those effects that have a long latency period for development (e.g., carcinogenicity and life shortening). It provides information on health hazards likely to arise from repeated exposures by the inhalation route over a limited period of time. It will provide information on target organs, the possibilities of accumulation, and can be of use in selecting dose levels for chronic studies and for establishing safety criteria for human exposure. Hazards of inhaled substances are influenced by the inherent toxicity and by physical factors such as volatility and particle size.

(b) *Definitions.* (1) Subchronic inhalation toxicity is the adverse effects occurring as a result of the repeated daily exposure of experimental animals to a chemical by inhalation for part (approximately 10 percent) of a life span.

(2) Aerodynamic diameter applies to the size of particles of aerosols. It is the diameter of a sphere of unit densi-

ty which behaves aerodynamically as the particle of the test substance. It is used to compare particles of different size and densities and to predict where in the respiratory tract such particles may be deposited. This term is used in contrast to measured or geometric diameter which is representative of actual diameters which in themselves cannot be related to deposition within the respiratory tract.

(3) The geometric mean diameter or the median diameter is the calculated aerodynamic diameter which divides the particles of an aerosol in half based on the weight of the particles. Fifty percent of the particles by weight will be larger than the median diameter and 50 percent of the particles will be smaller than the median diameter. The median diameter describes the particle size distribution of any aerosol based on the weight and size of the particles.

(4) Inhalable diameter refers to that aerodynamic diameter of a particle which is considered to be inhalable for the organism. It is used to refer to particles which are capable of being inhaled and may be deposited anywhere within the respiratory tract from the trachea to the alveoli. For man, inhalable diameter is considered as 15 micrometers or less.

(5) Dose refers to an exposure level. Exposure is expressed as weight or volume of test substance per volume of air (mg/l), or as parts per million (ppm).

(6) No-effect level/No-toxic-effect level/No-adverse-effect level/No-observed-effect level is the maximum dose used in a test which produces no observed adverse effects. A no-observed-effect level is expressed in terms of weight or volume of test substance given daily per unit volume of air (mg/l or ppm).

(7) Cumulative toxicity is the adverse effects of repeated doses occuring as a result of prolonged action on, or increased concentration of the administered test substance or its metabolites in susceptible tissues.

(c) *Principle of the test method.* Several groups of experimental animals are exposed daily for a defined period to the test substance in graduated concentrations, one concentration being used per group, for a period of 90 days. During the period of administration, the animals are observed daily to detect signs of toxicity. Animals which die during the test are necropsied and at the conclusion of the test, surviving animals are sacrificed and necropsied and appropriate histopathological examinations carried out.

(d) *Test procedures*—(1) *Animal selection*—(i) *Species and strain.* A mammalian species shall be used for testing. A variety of rodent species may be used, although the rat is the preferred species. Commonly used laboratory strains shall be employed. If another mammalian species is used, the tester shall provide justification/ reasoning for its selection.

(ii) *Age.* Young adult animals shall be used. At the commencement of the study the weight variation of animals shall not exceed ±20 percent of the mean weight for each sex.

(iii) *Sex.* (A) Equal numbers of animals of each sex shall be used at each dose level.

(B) Females shall be nulliparous and nonpregnant.

(iv) *Numbers.* (A) At least 20 rodents (10 females and 10 males) shall be used for each test group. If another mammalian species is selected (e.g. dog, rabbit, or non-human primate), at least 8 animals (4 males and 4 females) shall be used.

(B) If interim sacrifices are planned, the number of animals shall be increased by the number of animals scheduled to be sacrificed before the completion of the study.

(2) *Control groups.* A concurrent control group is required. This group shall be an untreated or sham-treated control group. Except for treatment with the test substance, animals in the control group shall be handled in a manner identical to the test group animals. Where a vehicle is used to help generate an appropriate concentration of the substance in the atmosphere, a vehicle control group shall be used. If the toxic properties of the vehicle are not known or cannot be made available, both untreated and vehicle control groups are required.

(3) *Satellite group.* A satellite group of 20 animals (10 animals per sex) may be treated with the high concentration

level for 90 days and observed for reversibility, persistence, or delayed occurrence of toxic effects for a posttreatment period of appropriate length, normally not less than 28 days.

(4) *Dose levels and dose selection.* (i) In subchronic toxicity tests, it is desirable to have a concentration-response relationship as well as a no-observed-toxic-effect level. Therefore, at least 3 concentration levels with a control and, where appropriate, a vehicle control (corresponding to the concentration of vehicle at the highest exposure level) shall be used. Concentrations should be spaced appropriately to produce test groups with a range of toxic effects. The data should be sufficient to produce a concentration-response curve.

(ii) The highest concentration should result in toxic effects but not produce an incidence of fatalities which would prevent a meaningful evaluation.

(iii) The lowest concentration should not produce any evidence of toxicity. Where there is a usable estimation of human exposure the lowest concentration should exceed this.

(iv) Ideally, the intermediate concentration level(s) should produce minimal observable toxic effects. If more than one intermediate concentration level is used, the concentrations should be spaced to produce a gradation of toxic effects.

(v) In the low and intermediate groups and in the controls the incidence of fatalities should be low, to permit a meaningful evaluation of the results.

(vi) In the case of potentially explosive test substances, care should be taken to avoid generating explosive concentrations.

(5) *Exposure conditions.* The animals should be exposed to the test substance, ideally for 6 hours per day on a 7-day per week basis, for a period of 90 days. However, based primarily on practical considerations, exposure on a 5-day-per-week basis for 6 hours per day is the minimum acceptable exposure period.

(6) *Observation period.* (i) Duration of observation shall be for at least 90 days.

(ii) Animals in a satellite group scheduled for followup observations should be kept for at least 28 days further without treatment to detect recovery from, or persistence of, toxic effects.

(7) *Inhalation exposure.* (i) The animals shall be tested in inhalation equipment designed to sustain a minimum dynamic air flow of 12 to 15 air changes per hour and ensure an adequate oxygen content of 19 percent and an evenly distributed exposure atmosphere. Where a chamber is used, its design should minimize crowding of the test animals and maximize their exposure to the test substance. This is best accomplished by individual caging. To ensure stability of a chamber atmosphere, the total "volume" of the test animals shall not exceed 5 percent of the volume of the test chamber. Oronasal or head-only exposure may be used if it is desirable to avoid concurrent exposure by the dermal or oral routes.

(ii) A dynamic inhalation system with a suitable flow control system shall be used. The rate of air flow shall be adjusted to ensure that conditions throughout the exposure chamber are essentially the same. Maintenance of slight negative pressure inside the chamber will prevent leakage of the test substance into surrounding areas.

(iii) The temperature at which the test is performed should be maintained at 22 °C (±2°). Ideally, the relative humidity should be maintained between 40 to 60 percent, but in certain instances (e.g., tests of aerosols, use of water vehicle) this may not be practicable.

(8) *Physical measurements.* Measurements or monitoring shall be made of the following:

(i) The rate of air flow shall be monitored continuously and recorded at least every 30 minutes.

(ii) The actual concentrations of the test substance shall be measured in the breathing zone. During the exposure period the actual concentrations of the test substance shall be held as constant as practicable, monitored continuously or intermittently depending on the method of analysis, and recorded at least at the beginning, at an intermediate time, and at the end of the exposure period.

(iii) During the development of the generating system, particle size analysis shall be performed to establish the stability of aerosol concentrations with respect to particle size. During exposure, analysis shall be conducted as often as necessary to determine the consistency of particle size distribution.

(iv) Temperature and humidity shall be monitored continuously but shall be recorded at least every 30 minutes.

(9) *Feed and water during exposure period.* Feed shall be withheld during exposure. Water may also be withheld during exposure.

(10) *Observation of animals.* (i) Each animal shall be observed daily and, if necessary, handled to appraise its physical condition.

(ii) Additional observations should be made daily with appropriate actions taken to minimize loss of animals to the study (e.g., necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals).

(iii) Signs of toxicity shall be recorded as they are observed including the time of onset, the degree, and duration.

(iv) Cage-side observations should include, but not be limited to, changes in the skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behavior pattern.

(v) Animals shall be weighed weekly. Feed consumption shall also be determined weekly if abnormal body weight changes are observed.

(vi) At the end of the study period all survivors in the nonsatellite treatment groups shall be sacrificed. Moribund animals shall be removed and sacrificed when noticed.

(11) *Clinical examinations.* (i) The following examinations shall be made on all animals of each sex in each group:

(A) Certain hematology determinations shall be carried out at least two times during the test period on all groups of animals including concurrent controls: After 30 days of test and just prior to terminal sacrifice at the end of the test period. Hematology determinations which are appropriate to all studies: Hematocrit, hemoglobin concentration, erythrocyte count, total and differential leukocyte count, and a measure of clotting potential such as clotting time, prothrombin time, thromboplastin time, or platelet count.

(B) Certain clinical biochemistry determinations on blood should be carried out at least two times during the test period on all groups of animals including concurrent controls: After 30 days of test and just prior to terminal sacrifice at the end of the test period. Clinical biochemistry test areas which are considered appropriate to all studies: Electrolyte balance, carbohydrate metabolism, and liver and kidney function. The selection of specific tests will be influenced by observations on the mode of action of the substance. Suggested determinations: calcium, phosphorus, chloride, sodium, potassium, fasting glucose (with period of fasting appropriate to the species), serum glutamic-pyruvic transaminase, (now known as serum alanine aminotransferase), serum glutamic-oxaloacetic transaminase (now known as serum aspartate aminotransferase), ornithine decarboxylase, gamma glutamyl transpeptidase, urea nitrogen, albumen, blood creatinine, total bilirubin, and total serum protein measurements. Other determinations which may be necessary for an adequate toxicological evaluation include: Analyses of lipids, hormones, acid/base balance, methemoglobin, and cholinesterase activity. Additional clinical biochemistry may be employed, where necessary, to extend the investigation of observed effects.

(ii) The following examinations shall be made on high dose and control groups. If changes in the eyes are detected, all animals shall be examined:

(A) Ophthalmological examination, using an ophthalmoscope or equivalent suitable equipment, shall be made prior to exposure to the test substance and at the termination of the study.

(B) Urinalysis is not recommended on a routine basis, but only when there is an indication based on expected and/or observed toxicity.

(12) *Gross pathology.* (i) All animals shall be subjected to a full gross necropsy which includes examination of the external surface of the body, all orifices and the cranial, thoracic, and abdominal cavities and their contents.

(ii) At least the liver, kidneys, adrenals, brain, and gonads shall be weighed wet, as soon as possible after dissection to avoid drying. In addition, for the rodent, the brain; for the nonrodent, the thyroid with parathyroids also shall be weighed wet.

(iii) The following organs and tissues, or representative samples thereof, shall be preserved in a suitable medium for possible future histopathological examination: All gross lesions; lungs—which should be removed intact, weighed, and treated with a suitable fixative to ensure that lung structure is maintained (perfusion with the fixative is considered to be an effective procedure); nasopharyngeal tissues; brain—including sections of medulla/pons cerebellar cortex and cerebral cortex; pituitary; thyroid/parathyroid; thymus; trachea; heart; sternum with bone marrow; salivary glands; liver; spleen; kidneys; adrenals; pancreas; gonads; uterus; accessory genital organs (epididymis, prostate, and, if present, seminal vesicles); aorta; (skin); gall bladder (if present); esophagus; stomach; duodenum; jejunum; ileum; cecum; colon; rectum; urinary bladder; representative lymph node; (mammary gland); (thigh musculature); peripheral nerve; (eyes); (femur—including articular surface); (spinal cord at three levels—cervical, midthoracic, and lumbar); and (zymbal and exorbital lachrymal glands).

(13) *Histopathology.* The following histopathology shall be performed:

(i) Full histopathology on the respiratory tract and other organs and tissues, listed above, of all animals in the control and high dose groups.

(ii) All gross lesions in all animals.

(iii) Target organs in all animals.

(iv) The tissues mentioned in brackets (listed above) if indicated by signs of toxicity or target organ involvement.

(v) Lungs of animals (rodents) in the low and intermediate dose groups shall also be subjected to histopathological examination, primarily for evidence of infection since this provides a convenient assessment of the state of health of the animals.

(vi) When a satellite group is used, histopathology shall be performed on tissues and organs identified as showing effects in the treated groups.

(e) *Data and reporting*—(1) *Treatment of results.* (i) Data shall be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions, the types of lesions, and the percentage of animals displaying each type of lesion.

(ii) All observed results, quantitative and incidental, should be evaluated by an appropriate statistical method. Any generally accepted statistical method may be used; the statistical methods should be selected during the design of the study.

(2) *Evaluation of results.* The findings of the subchronic inhalation toxicity study should be evaluated in conjunction with the findings of preceding studies and considered in terms of the observed toxic effects and the necropsy and histopathological findings. The evaluation will include the relationship between the concentration of the test substance and duration of exposure, and the presence or absence, the incidence and severity, of abnormalities, including behavioral and clinical abnormalities, gross lesions, identified target organs, body weight changes, effects on mortality and any other general or specific toxic effects. A properly conducted subchronic test should provide a satisfactory estimation of a no-effect level.

(3) *Test report.* In addition to the reporting requirements as specified under EPA Good Laboratory Practice Standards, 40 CFR Part 792, Subpart J, the following specific information shall be reported:

(i) *Test conditions.* (A) Description of exposure apparatus, including design, type, dimensions, source of air, system for generating particulates and aerosols, method of conditioning air, treatment of exhaust air, and the method of housing animals in a test chamber.

(B) The equipment for measuring temperature, humidity, and particulate aerosol concentrations and size shall be described.

(ii) *Exposure data.* These shall be tabulated and presented with mean values and measure of variability (e.g., standard deviation) and shall include:

(A) Airflow rates through the inhalation equipment.

(B) Temperature and humidity of air.

(C) Nominal concentration (total amount of test substance fed into the inhalation equipment divided by volume of air).

(D) Actual concentration in test breathing zone.

(E) Particle size distribution (e.g., median aerodynamic diameter of particles with standard deviation from the mean).

(iii) *Group animal data.* Tabulation of toxic response data by species, strain, sex, and exposure level for:

(A) Number of animals dying.

(B) Number of animals showing signs of toxicity.

(C) Number of animals exposed.

(iv) *Individual animal data.* (A) Date of death during the study or whether animals survived to termination.

(B) Date of observation of each abnormal sign and its subsequent course.

(C) Body weight data.

(D) Feed consumption data when collected.

(E) Hematological tests employed and all results.

(F) Clinical biochemistry tests employed and all results.

(G) Necropsy findings.

(H) Detailed description of all histopathological findings.

(I) Statistical treatment of results where appropriate.

(f) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Cage, J.C. "Experimental Inhalation Toxicology," *Methods in Toxicology.* Ed. G.E. Paget. (Philadelphia: F.A. Davis Co. 1970, pp. 258–277.

(2) Casarett, L.J., Doull, J. "Chapter 9." *Toxicology: The Basic Science of Poisons* (New York: Macmillan Publishing Co. Inc. 1975).

(3) MacFarland, H.N. "Respiratory Toxicology," *Essays in Toxicology.* Ed. W.J. Hayes. Vol. 7 (New York: Academic Press, 1976) pp. 121–154.

(4) National Academy of Sciences. "Principles and Procedures for Evaluating the Toxicity of Household Substances," a report prepared by the Committee for the Revision of NAS Publication 1138, under the auspices of the Committee on Toxicology, National Research Council, National Academy of Sciences, Washington, DC (1977).

(5) World Health Organization. "Part I. Environmental Health Criteria 6," *Principles and Methods for Evaluating the Toxicity of Chemicals.* (Geneva: World Health Organization, 1978).

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### § 798.2650 Oral toxicity.

(a) *Purpose.* In the assessment and evaluation of the toxic characteristics of a chemical, the determination of subchronic oral toxicity may be carried out after initial information on toxicity has been obtained by acute testing. The subchronic oral study has been designed to permit the determination of the no-observed-effect level and toxic effects associated with continuous or repeated exposure to a test substance for a period of 90 days. The test is not capable of determining those effects that have a long latency period for development (e.g., carcinogenicity and life shortening). It provides information on health hazards likely to arise from repeated exposure by the oral route over a limited period of time. It will provide information on target organs, the possibilities of accumulation, and can be of use in selecting dose levels for chronic studies and for establishing safety criteria for human exposure.

(b) *Definitions.*(1) Subchronic oral toxicity is the adverse effects occurring as a result of the repeated daily exposure of experimental animals to a chemical by the oral route for a part (approximately 10 percent) of a life span.

(2) Dose is the amount of test substance administered. Dose is expressed

as weight of test substance (g, mg) per unit weight of test animal (e.g., mg/kg), or as weight of test substance per unit weight of food or drinking water.

(3) No-effect level/No-toxic-effect level/No-adverse-effect level/No-observed-effect level is the maximum dose used in a test which produces no observed adverse effects. A no-observed-effect level is expressed in terms of the weight of a substance given daily per unit weight of test animal (mg/kg). When administered to animals in food or drinking water the no-observed-effect level is expressed as mg/kg of food or mg/ml of water.

(4) Cumulative toxicity is the adverse effects of repeated doses occurring as a result of prolonged action on, or increased concentration of, the administered test substance or its metabolites in susceptible tissue.

(c) *Principle of the test method.* The test substance is administered orally in graduated daily doses to several groups of experimental animals, one dose level per group, for a period of 90 days. During the period of administration the animals are observed daily to detect signs of toxicity. Animals which die during the period of administration are necropsied. At the conclusion of the test all animals are necropsied and histo-pathological examinations carried out.

(d) *Limit test.* If a test at one dose level of at least 1,000 mg/kg body weight (expected human exposure may indicate the need for a higher dose level), using the procedures described for this study, produces no observable toxic effects and if toxicity would not be expected based upon data of structurally related compounds, then a full study using three dose levels might not be necessary.

(e) *Test procedures*—(1) *Animal selection*—(i) *Species and strain.* A mammalian species shall be used for testing. A variety of rodent species may be used, although the rat is the preferred species. Commonly used laboratory strains shall be employed. The commonly used nonrodent species is the dog, preferably of a defined breed; the beagle is frequently used. If other mammalian species are used, the tester shall provide justification/reasoning for his or her selection.

(ii) *Age*—(A) *General.* Young adult animals shall be employed. At the commencement of the study the weight variation of animals used shall not exceed ± 20 percent of the mean weight for each sex.

(B) *Rodents.* Dosing shall begin as soon as possible after weaning, ideally before the rats are 6, and in any case, not more than 8 weeks old.

(C) *Non-rodent.* In the case of the dog, dosing shall commence after acclimatization, preferably at 4 to 6 months and not later than 9 months of age.

(iii) *Sex.* (A) Equal numbers of animals of each sex shall be used at each dose level.

(B) The females shall be nulliparous and nonpregnant.

(iv) *Numbers*—(A) *Rodents.* At least 20 animals (10 females and 10 males) shall be used at each dose level.

(B) *Non-rodents.* At least eight animals (four females and four males) shall be used at each dose level.

(C) If interim sacrifices are planned, the number shall be increased by the number of animals scheduled to be sacrificed before the completion of the study.

(2) *Control groups.* A concurrent control group is required. This group shall be an untreated or sham-treated control group or, if a vehicle is used in administering the test substance, a vehicle control group. If the toxic properties of the vehicle are not known or cannot be made available, both untreated and vehicle control groups are required.

(3) *Satellite group.* (Rodent) A satellite group of 20 animals (10 animals per sex) may be treated with the high dose level for 90 days and observed for reversibility, persistence, or delayed occurrence of toxic effects for a posttreatment period of appropriate length, normally not less than 28 days.

(4) *Dose levels and dose selection.* (i) In subchronic toxicity tests, it is desirable to have a dose response relationship as well as a no-observed-toxic-effect level. Therefore, at least 3 dose levels with a control and, where appropriate, a vehicle control (corresponding to the concentration of vehicle at

the highest exposure level) shall be used. Doses should be spaced appropriately to produce test groups with a range of toxic effects. The data should be sufficient to produce a dose-response curve.

(ii) The highest dose level in rodents should result in toxic effects but not produce an incidence of fatalities which would prevent a meaningful evaluation; for non-rodents there should be no fatalities.

(iii) The lowest dose level should not produce any evidence of toxicity. Where there is a usable estimation of human exposure the lowest dose level should exceed this.

(iv) Ideally, the intermediate dose level(s) should produce minimal observable toxic effects. If more than one intermediate dose is used, the dose levels should be spaced to produce a gradation of toxic effects.

(v) For rodents, the incidence of fatalities in low and intermediate dose groups and in the controls should be low, to permit a meaningful evaluation of the results; for non-rodents, there should be no fatalities.

(5) *Exposure conditions.* The animals are dosed with the test substance ideally on a 7-day per week basis over a period of 90 days. However, based primarily on practical considerations, dosing in gavage or capsule studies on a 5-day per week basis is considered to be acceptable.

(6) *Observation period.* (i) Duration of observation shall be for at least 90 days.

(ii) Animals in the satellite group scheduled for followup observations should be kept for at least 28 days further without treatment to detect recovery from, or persistence of, toxic effects.

(7) *Administration of the test substance.* (i) The test substance may be administered in the diet or in capsules. In addition, for rodents it may also be administered by gavage or in the drinking water.

(ii) All animals shall be dosed by the same method during the entire experimental period.

(iii) Where necessary, the test substance is dissolved or suspended in a suitable vehicle. If a vehicle or diluent is needed, ideally it should not elicit important toxic effects itself nor substantially alter the chemical or toxicological properties of the test substance. It is recommended that wherever possible the usage of an aqueous solution be considered first, followed by consideration of a solution of oil and then by possible solution in other vehicles.

(iv) For substances of low toxicity, it is important to ensure that when administered in the diet the quantities of the test substance involved do not interfere with normal nutrition. When the test substance is administered in the diet either a constant dietary concentration (ppm) or a constant dose level in terms of the animals' body weight shall be used; the alternative used shall be specified.

(v) For a substance administered by gavage or capsule, the dose shall be given at approximately the same time each day, and adjusted at intervals (weekly or bi-weekly) to maintain a constant dose level in terms of animal body weight.

(8) *Observation of animals.* (i) Each animal shall be observed daily and, if necessary, handled to appraise its physical condition.

(ii) Additional observations shall be made daily with appropriate actions taken to minimize loss of animals to the study (e.g., necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals).

(iii) Signs of toxicity shall be recorded as they are observed including the time of onset, degree and duration.

(iv) Cage-side observations shall include, but not be limited to, changes in skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behavior pattern.

(v) Measurements shall be made weekly of feed consumption or water consumption when the test substance is administered in the feed or drinking water, respectively.

(vi) Animals shall be weighed weekly.

(vii) At the end of the 90-day period all survivors in the nonsatellite treatment groups shall be sacrificed. Moribund animals shall be removed and sacrificed when noticed.

(9) *Clinical examinations.* (i) The following examinations shall be made on all animals of each sex in each group for rodents and all animals when non-rodents are used as test animals.

(A) Certain hematology determinations shall be carried out at least two times during the test period on all groups of animals including concurrent controls: After 30 days of test and just prior to terminal sacrifice at the end of the test period. Hematology determinations which are appropriate to all studies: Hematocrit, hemoglobin concentration, erythrocyte count, total and differential leukocyte count, and a measure of clotting potential such as clotting time, prothrombin time, thromboplastin time, or platelet count.

(B) Certain clinical biochemistry determinations on blood should be carried out at least two times during the test period on all groups of animals including concurrent controls: After 30 days of test and just prior to terminal sacrifice at the end of the test period. Clinical biochemistry test areas which are considered appropriate to all studies: Electrolyte balance, carbohydrate metabolism, and liver and kidney function. The selection of specific tests will be influenced by observations on the mode of action of the substance. Suggested determinations: Calcium, phosphorus, chloride, sodium, potassium, fasting glucose (with period of fasting appropriate to the species), serum glutamic-pyruvic transaminase (now known as serum alanine aminotransferase), serum glutamic oxaloacetic transaminase (now known as serum aspartate aminotransferase), ornithine decarboxylase, gamma glutamyl transpeptidase, urea nitrogen, albumen, blood creatinine, total bilirubin, and total serum protein measurements. Other determinations which may be necessary for an adequate toxicological evaluation include: Analyses of lipids, hormones, acid/base balance, methemoglobin, and cholinesterase activity. Additional clinical biochemistry may be employed, where necessary, to extend the investigation of observed effects.

(ii) The following examinations shall be made on high dose and control groups. If changes in the eyes are detected, all animals should be examined.

(A) Ophthalmological examination, using an ophthalmoscope or equivalent suitable equipment, shall be made prior to the administration of the test substance and at the termination of the study.

(B) Urinalysis is not recommended on a routine basis, but only when there is an indication based on expected and or observed toxicity.

(10) *Gross necropsy.* (i) All animals shall be subjected to a full gross necropsy which includes examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents.

(ii) At least the liver, kidneys, adrenals, and gonads shall be weighed wet, as soon as possible after dissection to avoid drying. In addition, for the rodent, the brain; for the non-rodent, the thyroid with parathyroids also shall be weighed wet.

(iii) The following organs and tissues, or representative samples thereof, shall be preserved in a suitable medium for possible future histopathological examination: All gross lesions; lungs—which should be removed intact, weighed, and treated with a suitable fixative to ensure that lung structure is maintained (perfusion with the fixative is considered to be an effective procedure); nasopharyngeal tissues; brain— including sections of medulla/pons, cerebellar cortex, and cerebral cortex; pituitary; thyroid/parathyroid; thymus; trachea; heart; sternum with bone marrow; salivary glands; liver; spleen; kidneys; adrenals; pancreas; gonads; uterus; accessory genital organs (epididymis, prostate, and, if present, seminal vesicles); aorta; (skin); gall bladder (if present); esophagus; stomach; duodenum; jejunum; ileum; cecum; colon; rectum; urinary bladder; representative lymph node; (mammary gland); (thigh musculature); peripheral nerve; (eyes); (femur—including articular surface); (spinal cord at three levels—cervical, midthoracic, and lumbar); and (zymbal and exorbital lachrymal glands); and (rodent-zymbal glands).

(11) *Histopathology.* The following histopathology shall be performed:

(i) Full histopathology on the organs and tissues, listed above, of all rodents in the control and high dose groups, all non-rodents, and all rodents that died or were killed during the study.

(ii) All gross lesions in all animals.

(iii) Target organs in all animals.

(iv) The tissues mentioned in brackets (listed above) if indicated by signs of toxicity of target organ involvement.

(v) Lungs, liver and kidneys of all animals. Special attention to examination of the lungs of rodents shall be made for evidence of infection since this provides a convenient assessment of the state of health of the animals.

(vi) When a satellite group is used (rodents), histopathology shall be performed on tissues and organs identified as showing effects in the treated groups.

(f) *Data and reporting*—(1) *Treatment of results.* (i) Data shall be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions, the types of lesions and the percentage of animals displaying each type of lesion.

(ii) All observed results, quantitative and incidental, should be evaluated by an appropriate statistical method. Any generally accepted statistical methods may be used; the statistical methods should be selected during the design of the study.

(2) *Evaluation of the study results.* (i) The findings of a subchronic oral toxicity study should be evaluated in conjunction with the findings of preceding studies and considered in terms of the toxic effects and the necropsy and histopathological findings. The evaluation will include the relationship between the dose of the test substance and the presence or absence, the incidence and severity, of abnormalities, including behavioral and clinical abnormalities, gross lesions, identified target organs, body weight changes, effects on mortality and any other general or specific toxic effects. A properly conducted subchronic test should provide a satisfactory estimation of a no-effect level.

(ii) In any study which demonstrates an absence of toxic effects, further investigation to establish absorption and bioavailability of the test substance should be considered.

(3) *Test report.* In addition to the reporting requirements as specified under EPA Good Laboratory Practice Standards, 40 CFR Part 792, Subpart J, the following specific information shall be reported:

(i) *Group animal data.* Tabulation of toxic response data by species, strain, sex and exposure level for:

(A) Number of animals dying.

(B) Number of animals showing signs of toxicity.

(C) Number of animals exposed.

(ii) *Individual animal data.* (A) Date of death during the study or whether animals survived to termination.

(B) Date of observation of each abnormal sign and its subsequent course.

(C) Body weight data.

(D) Feed consumption data when collected.

(E) Hematological tests employed and all results.

(F) Clinical biochemistry tests employed and all results.

(G) Necropsy findings.

(H) Detailed description of all histopathological findings.

(I) Statistical treatment of results where appropriate.

(g) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Boyd, E.M. "Chapter 14—Pilot Studies, 15—Uniposal Clinical Parameters, 16—Uniposal Autopsy Parameters." *Predictive Toxicometrics.* (Baltimore: Williams and Wilkins, 1972).

(2) Fitzhugh, O.G. "Subacute Toxicity," *Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics.* The Association of Food and Drug Officials of the United States (1959, 3rd Printing 1975) pp. 26–35.

(3) Food Safety Council. "Subchronic Toxicity Studies," *Proposed System for Food Safety Assessment.* (Columbia: Food Safety Council, 1978) pp. 83–96.

(4) National Academy of Sciences. "Principles and Procedures for Evaluating the Toxicity of Household Sub-

stances," a report prepared by the Committee for the Revision of NAS Publication 1138, under the auspices of the Committee on Toxicology, National Research Council, National Academy of Sciences, Washington, DC (1977).

(5) World Health Organization. "Part I. Environmental Health Criteria 6," *Principles and Methods for Evaluating the Toxicity of Chemicals.* (Geneva: World Health Organization, 1978).

[50 FR 39397, Sept. 27, 1985, as amended at 52 FR 19074, May 20, 1987; 53 FR 49150, Dec. 6, 1988; 54 FR 21064, May 16, 1989]

### § 798.2675 Oral toxicity with satellite reproduction and fertility study.

(a) *Purpose.* (1) In the assessment and evaluation of the toxic characteristics of a chemical, the determination of subchronic oral toxicity may be carried out after initial information on toxicity has been obtained by acute testing. The subchronic oral study has been designed to permit the determination of the no-observed-effect level and toxic effects associated with continuous or repeated exposure to a test substance for a period of 90 days. The test is not capable of determining those effects that have a long latency period for development (e.g., carcinogenicity and life shortening). It provides information on health hazards likely to arise from repeated exposure by the oral route over a limited period of time. It will provide information on target organs, and on the possibilities of accumulation, and can be of use in selecting dose levels for chronic studies and for establishing safety criteria for human exposure.

(2) The satellite reproduction and fertility study is designed to provide general information concerning the effects of a test substance on gonadal function, conception, parturition, and the growth and development of the offspring. The study may also provide information about the effects of the test substance on neonatal morbidity, mortality, and preliminary data on teratogenesis and serve as a guide for subsequent tests.

(b) *Definitions.* (1) Subchronic oral toxicity is the adverse effects occurring as a result of the repeated daily exposure of experimental animals to a chemical by the oral route for a part (approximately 10 percent for rats) of a life span.

(2) Dose is the amount of test substance administered. Dose is expressed as weight of test substance (g, mg) per unit weight of test animal (e.g., mg/kg), or as weight of test substance per unit weight of food or drinking water.

(3) No-effect level/No-toxic-effect level/No-adverse-effect level/No-observed effect level is the maximum dose used in a test which produces no-observed-adverse-effects. A no-observed effect level is expressed in terms of the weight of a substance given daily per unit weight of test animal (mg/kg). When administered to animals in food or drinking water the no-observed-effect level is expressed as mg/kg of food or mg/ml of water.

(4) Cumulative toxicity is the adverse effects of repeated doses occurring as a result of prolonged action on, or increased concentration of the administered substance or its metabolites in susceptible tissue.

(c) *Principle of the test method.* (1) The test substance is administered orally in graduated daily doses to several groups of experimental animals, one dose level per group, for a period of 90 days for the subchronic study. During the period of administration the animals are observed daily to detect signs of toxicity. Animals which die during the period of administration are necropsied. At the conclusion of the test all surviving animals are necropsied and histopathological examinations carried out.

(2) Starting with the 10th week of dosing, males will be mated with females selected for the reproduction and fertility study. The test substance will continue to be administered throughout the period of mating until day 90 for all males and those females not selected for reproduction; for the mated females, dosing will continue throughout the resulting period of gestation and lactation until weaning of their offspring.

(d) *Limit test.* If a test at one dose level of at least 1,000 mg/kg body weight (expected human exposure may indicate the need for a higher dose level), using the procedures described for this study, produces no observable toxic effects and if toxicity would not be expected based upon data of structurally related compounds, then a full study using three dose levels might not be necessary.

(e) *Test procedures*—(1) *Animal selection*—(i) *Species and strain.* The rat is the preferred species. If another mammalian species is used, the tester should provide justification/reasoning for its selection. Strains with low fecundity should not be used.

(ii) *Age.* Animals should be about 5 to 8 weeks old at the start of dosing.

(iii) *Sex.* (A) Both males and females should be studied.

(B) The females should be nulliparous and non-pregnant.

(iv) *Number of animals.* Each test and control group should contain at least 10 males and at least 30 females (at least 10 for subchronic study and a sufficient amount of additional females to yield at least 20 pregnancies for the reproduction and fertility study).

(2) *Control groups.* (i) A concurrent control group should be used. This group should be an untreated or sham treated control group or if a vehicle is used in administering the test substance, a vehicle control group.

(ii) If a vehicle is used in administering the test substance, the control group should receive the vehicle in the highest volume used.

(iii) If a vehicle or other additive is used to facilitate dosing, it should not interfere with absorption of the test substance or produce toxic effects.

(3) *Dose levels and dose selection.* (i) In subchronic toxicity tests, it is desirable to have a dose response relationship as well as no-observed-toxic-effect level. Therefore, at least three dose levels with a control and, where appropriate, a vehicle control (corresponding to the concentration of vehicle at the highest exposure level) should be used. Doses should be spaced appropriately to produce test groups with a range of toxic effects. The data should be sufficient to produce a dose response curve.

(ii) The highest dose level in rodents should result in toxic effects but not produce an incidence of fatalities which would prevent a meaningful evaluation.

(iii) The lowest dose level should not produce any evidence of toxicity. Where there is a usable estimation of human exposure the lowest dose level should exceed this.

(iv) Ideally, the intermediate dose level(s) should produce minimal observable toxic effects. If more than one intermediate dose is used, the dose levels should be spaced to produce a gradation of toxic effects.

(4) *Exposure conditions.* The animals are dosed with the test substance ideally on a 7-day per week basis over a period of 90 days. However, based primarily on practical considerations, dosing in gavage or capsule studies on a 5-day per week basis is considered to be acceptable, except during the periods of mating, gestation, and lactation during which time females selected for the reproduction and fertility study should be dosed daily.

(i) Dosing, mating, delivery, and sacrifice schedule.

(A) Dosing of the males and females should begin when they are 5 to 8 weeks old. For both sexes, dosing should be continued for at least 10 weeks before the mating period.

(B) Dosing of males should continue through the mating period and until day 90 of the subchronic study.

(C) Dosing of females not selected for the reproduction and fertility study should continue until day 90 of the subchronic study.

(D) Daily dosing (that is, on a 7-day per week basis) of the females selected for the reproduction and fertility study should begin with and continue through the mating period, pregnancy, and to the weaning of the offspring.

(ii) All animals are sacrificed as scheduled.

(A) All males and those females not selected for reproduction and fertility study should be sacrificed at the end of the 90-day subchronic study.

(B) The females selected for the reproduction and fertility study should be sacrificed upon weaning of their offspring.

(C) Offspring should be sacrificed when weaned.

(5) *Observation period.* (i) Duration of observation should be for at least 90 days for the subchronic study.

(ii) For the satellite reproduction and fertility study, observation of dams and their pups should continue until the weaning of the offspring.

(6) *Administration of the test substance.* (i) Test substance may be administered in the diet or in capsules. In addition, for rodents it may also be administered by gavage or in the drinking water.

(ii) All animals should be dosed by the same method during the entire experimental period.

(iii) Where necessary, the test substance is dissolved or suspended in a suitable vehicle. If a vehicle or diluent is needed, ideally it should not elicit important toxic effects itself nor substantially alter the chemical or toxicological properties of the test substance. It is recommended that wherever possible the usage of an aqueous solution be considered first, followed by consideration of a solution of oil and then by possible solution in other vehicles.

(iv) For substances of low toxicity, it is important to ensure that when administered in the diet the quantities of the test substance involved do not interfere with normal nutrition. When the test substance is administered in the diet either a constant dietary concentration (ppm) or a constant dose level in terms of the animals' body weight may be used; the alternative used should be specified.

(v) For a substance administered by gavage or capsule, the dose should be given at similar times each day and adjusted to maintain a constant dose level in terms of animal body weight. During pregnancy the dosage should be based on the body weight at day 0 and 6 of pregnancy.

(7) *Mating procedure*—(i) *Parental.* (A) For each mating, either a 1:1 (1 male to 1 female) or a 1:2 (1 male to 2 females) method may be used. Females should be placed with males from the same dose level until pregnancy occurs or 1 week has elapsed. If mating has not occurred after 1 week, the female should be placed with a different male. Paired matings should be clearly identified.

(B) Those pairs that fail to mate should be evaluated to determine the cause of the apparent infertility. This may involve such procedures as additional opportunities to mate with proven fertile males or females, histological examination of the reproductive organs, and examination of the estrus or spermatogenic cycles.

(C) Each day, the females should be examined for presence of sperm or vaginal plugs. Day 0 of pregnancy is defined as the day vaginal plugs or sperm are found.

(ii) *Special housing.* After evidence of copulation, pregnant animals should be caged separately in delivery or maternity cages and provided with nesting materials.

(iii) *Standardization of litter sizes.* (A) On day 4 after birth, the size of each litter should be adjusted by eliminating extra pups by random selection to yield, as nearly as possible, four males and four females per litter.

(B) Whenever the number of male or female pups prevents having four of each sex per litter, partial adjustment (for example, five males and three females) is permitted. Adjustments are not appropriate for litters of less than eight pups.

(C) Elimination of runts only is not appropriate.

(8) *Observation of animals.* (i) Each animal should be handled and its physical condition appraised at least once each day.

(ii) Additional observations should be made daily with appropriate actions taken to minimize loss of animals to the study (e.g., necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals).

(iii) Signs of toxicity should be recorded as they are observed including the time of onset, degree and duration.

(iv) Cage-side observations should include, but not be limited to, changes in skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behavior pattern.

(v) Measurements should be made weekly of feed consumption or water consumption when the test substance is administered in the feed or drinking water, respectively.

(vi) Animals should be weighed weekly.

(vii) At the end of the 90-day period all survivors in the non-satellite treatment groups are sacrificed. Moribund animals should be removed and sacrificed when noticed.

(viii) The duration of gestation should be calculated from day 0 of pregnancy. Signs of difficult or prolonged parturition should be recorded.

(ix) Each litter should be examined as soon as possible after delivery for the number of pups, stillbirths, live births, sex, and the presence of gross anomalies. Dead pups and pups sacrificed at day 4 should be preserved and studied for possible defects. Live pups should be counted and litters weighed, by weighing each individual pup at birth, or soon thereafter, and on days 4, 7, 14, and 21 after parturition.

(x) Physical or behavioral abnormalities observed in the dams or offspring should be recorded.

(xi) Males and females in subchronic and satellite studies should be weighed on the first day of dosing and weekly thereafter. Litters should be weighed at birth, or soon thereafter, and on days 4, 7, 14, and 21. In all cases, litter weights should be calculated from the weights of the individual pups.

(9) *Clinical examinations.* (i) The following examinations should be made on at least five animals of each sex in each group from the subchronic study.

(A) Certain hematology determinations should be carried out at least three times during the test period: just prior to initiation of dosing (baseline data), after approximately 30 days on test and just prior to terminal sacrifice at the end of the test period. Hematology determinations which should be appropriate to all studies: hematocrit, hemoglobin concentration, erythrocyte count, total and differential leucocyte count, and a measure of clotting potential such as clotting time, prothrombin time, thromboplastin time, or platelet count.

(B) Certain clinical biochemistry determinations should be carried out at least three times during the test period: just prior to initiation of dosing (baseline data), after approximately 30 days on test and just prior to terminal sacrifice at the end of the test period. Clinical biochemical test areas which are considered appropriate to all studies: electrolyte balance, carbohydrate metabolism, and liver and kidney function. The selection of specific tests will be influenced by observations on the mode of action of the substance. Suggested determinations: calcium, phosphorus, chloride, sodium, potassium, fasting glucose (with period of fasting appropriate to the species/breed), serum glutamic-pyruvic transaminase (now known as serum alanine aminotransferase), serum glutamic oxaloacetic transaminase (now known as serum aspartate aminotransferase), ornithine decarboxylase, gamma glutamyl transpeptidase, urea nitrogen, albumen, blood creatinine, total bilirubin and total serum protein measurements. Other determinations which may be necessary for an adequate toxicological evaluation include analyses of lipids, hormones, acid/base balance, methemoglobin and cholinesterase activity. Additional clinical biochemistry may be employed where necessary to extend the investigation of observed effects.

(ii) The following examinations should be made on at least five animals of each sex in each group.

(A) Ophthalmological examination, using an ophthalmoscope or equivalent suitable equipment, should be made prior to the administration of the test substance and at the termination of the study. If changes in the eyes are detected all animals should be examined.

(B) Urinalysis is not recommended on a routine basis, but only when there is an indication based on expected and/or observed toxicity.

(10) *Gross necropsy.* (i) All animals (except offspring) should be subjected to a full gross necropsy which includes examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents.

(ii) Special attention should be directed to the organs of the reproductive system.

(iii) At least the liver, kidneys, adrenals, brain and gonads should be weighed wet, as soon as possible after dissection to avoid drying.

(iv) The following organs and tissues, or representative samples thereof, should be preserved in a suitable medium for possible future histopathological examination: All gross lesions; brain-including sections of medulla/pons, cerebellar cortex and cerebral cortex; pituitary; thyroid/parathyroid; thymus; lungs; trachea; heart; sternum with bone marrow; salivary glands; liver; spleen; kidneys/adrenals; pancreas; vagina; uterus; cervix; ovaries; testes; epididymides; prostate; seminal vesicles; aorta; (skin); esophagus; stomach; duodenum; jejunum; ileum; cecum; colon; rectum; urinary bladder; representative lymph node; (mammary gland); (thigh musculature); peripheral nerve; (eyes); (femur including articular surface); (spinal cord at three levels—cervical, midthoracic and lumbar); and, (exorbital lachrymal glands).

(v) Dead or moribund pups should be examined for defects.

(11) *Histopathology*. The following histopathology should be performed:

(i) Full histopathology on the organs and tissues, listed above, of all animals (except offspring) in the control and high dose groups, and all animals (except offspring) that died or were killed during the study.

(ii) All gross lesions in all animals.

(iii) Target organs in all animals.

(iv) The tissues mentioned in brackets (listed above) if indicated by signs of toxicity or target organ involvement.

(v) Lungs, liver and kidneys of all animals. Special attention to examination of the lungs of rodents should be made for evidence of infection since this provides a convenient assessment of the state of health of the animals.

(vi) As suggested under mating procedures, reproductive organs of animals suspected of infertility may be subjected to microscopic examination.

(f) *Data and reporting*—(1) *Treatment of results*. (i) For the subchronic study, data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions, the types of lesions and the percentage of animals displaying each type of lesion.

(ii) For the reproduction and fertility study, data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals pregnant, the types of change and the percentage of animals displaying each type of change.

(iii) All observed results, quantitative and incidental, should be evaluated by an appropriate statistical method. Any generally accepted statistical methods may be used; the statistical methods should be selected during the design of the study.

(2) *Evaluation of the study results*. (i) The findings of a subchronic oral toxicity study should be evaluated in conjunction with the findings of preceding studies and considered in terms of the toxic effects and the necropsy and histopathological findings. The evaluation will include the relationship between the dose of the test substance and the presence or absence, the incidence and severity, of abnormalities, including behavioral and clinical abnormalities, gross lesions, identified target organs, body weight changes, effects on mortality and any other general or specific toxic effects. A properly conducted test should provide a satisfactory estimation of a no-effect level.

(ii) In any study which demonstrates an absence of toxic effects, further investigation to establish absorption and bioavailability of the test substance should be considered.

(3) *Test report*. In addition to the reporting requirements as specified under 40 CFR Part 792, Subpart J the following specific information should be reported:

(i) Tabulation of toxic response data by species, strain, sex, and exposure level for:

(A) Number of animals dying.

(B) Number of animals showing signs of toxicity.

(C) Number of animals exposed.

(ii) *Individual animal data*. (A) Time of death during the study or whether animals survived to termination.

(B) Time of observation of each abnormal sign and its subsequent course.

(C) Body weight data.

(D) Feed consumption data when collected.

(E) Hematological tests employed and all results.

(F) Clinical biochemistry tests employed and all results.

(G) Necropsy findings.

(H) Detailed description of all histopathological findings.

(I) Statistical treatment of results where appropriate.

(iii) *Additional information from reproduction and fertility satellite study.* (A) Toxic response data by sex and dose, including fertility, gestation, viability and lactation indices, and length of gestation.

(B) Species and strain.

(C) Toxic or other effects on reproduction, offspring, or postnatal growth.

(D) Time of observation of each abnormal sign and its subsequent course.

(E) Body weight data for parental animals and offspring.

(g) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Boyd, E.M. "Chapter 14—Pilot Studies, 15—Uniposal Clinical Parameters, 16—Uniposal Autopsy Parameters," *Predictive Toxicometrics.* (Baltimore: Williams and Wilkins, 1972).

(2) Clermont, Y., Perry, B. "Quantitative Study of the Cell Population of the Seminiferous Tubules in Immature Rats," *American Journal of Anatomy.* 100:241-267 (1957).

(3) Fitzhugh, O.C. Third Printing: 1975. "Subacute Toxicity," *Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics.* The Association of Food and Drug Officials of the United States (1959), pp. 26-35.

(4) Food Safety Council. "Subchronic Toxicity Studies," *Proposed System for Food Safety Assessment.* (Columbia: Food Safety Council, 1978) pp. 83-96.

(5) Goldenthal, E.I. *Guidelines for Reproduction Studies for Safety Evaluation of Drugs for Human Use.* Drug Review Branch, Division of Toxicological Evaluation, Bureau of Science, Food and Drug Administration, Washington, DC (1966).

(6) Hasegawa, T., Hayashi, M., Ebling, F.J.G., Henderson, I.W. *Fertility and Sterility.* (New York: American Elsevier Publishing Co., Inc., 1973).

(7) National Academy of Sciences. "Principles and Procedures for Evaluating the Toxicity of Household Substances," a report prepared by the Committee for the Revision of NAS Publication 1138, under the auspices of the Committee on Toxicology, National Research Council, National Academy of Sciences, Washington, DC (1977).

(8) Oakberg, E.F. "Duration of Spermatogenesis in the Mouse and Timing of Stages of the Cycle of the Seminiferous Epithelium," *American Journal of Anatomy.* 9:507-516 (1956).

(9) Roosen-Runge, E.C. "The Process of Spermatogenesis in Mammals," *Biological Review.* 37:343-377 (1962).

(10) World Health Organization. "Part I. Environmental Health Criteria 6," *Principles and Methods for Evaluating the Toxicity of Chemicals.* (Geneva: World Health Organization, 1978).

[50 FR 39397, Sept. 27, 1985, as amended at 54 FR 21064, May 16, 1989]

## Subpart D—Chronic Exposure

### § 798.3260 Chronic toxicity.

(a) *Purpose.* The objective of a chronic toxicity study is to determine the effects of a substance in a mammalian species following prolonged and repeated exposure. Under the conditions of the chronic toxicity test, effects which require a long latency period or which are cumulative should become manifest. The application of this guideline should generate data on which to identify the majority of chronic effects and shall serve to define long term dose-response relationships. The design and conduct of chronic toxicity tests should allow for the detection of general toxic effects, including neurological, physiological, biochemical, and hematological effects and exposure-related morphological (pathology) effects.

(b) *Test procedures*—(1) *Animal selection*—(i) *Species and strain.* Testing should be performed with two mammalian species, one a rodent and another a non-rodent. The rat is the preferred rodent species and the dog is the preferred non-rodent species. Commonly used laboratory strains should be employed. If other mammalian species are used, the tester should provide justification/reasoning for their selection.

(ii) *Age.* (A) Dosing of rats should begin as soon as possible after weaning, ideally before the rats are 6, but in no case more than 8 weeks old.

(B) Dosing of dogs should begin between 4 and 6 months of age and in no case later than 9 months of age.

(C) At commencement of the study the weight variation of animals used should not exceed ±20 percent of the mean weight for each sex.

(iii) *Sex.* (A) Equal numbers of animals of each sex should be used at each dose level.

(B) The females should be nulliparous and non-pregnant.

(iv) *Numbers.* (A) For rodents, at least 40 animals (20 females and 20 males) and for non-rodents (dogs) at least 8 animals (4 females and 4 males) should be used at each dose level.

(B) If interim sacrifices are planned, the number should be increased by the number of animals scheduled to be sacrificed during the course of the study.

(C) The number of animals at the termination of the study must be adequate for a meaningful and valid statistical evaluation of chronic effects.

(2) *Control groups.* (i) A concurrent control group is suggested. This group should be an untreated or sham treated control group or, if a vehicle is used in administering the test substance, a vehicle control group. If the toxic properties of the vehicle are not known or cannot be made available, both untreated and vehicle control groups are strongly suggested.

(ii) In special circumstances such as in inhalation studies involving aerosols or the use of an emulsifier of uncharacterized biological activity in oral studies, a concurrent negative control group should be utilized. The negative control group should be treated in the same manner as all other test animals except that this control group should not be exposed to either the test substance or any vehicle.

(3) *Dose levels and dose selections.* (i) In chronic toxicity tests, it is necessary to have a dose-response relationship as well as a no-observed-toxic-effect level. Therefore, at least three dose levels should be used in addition to the concurrent control group. Dose levels should be spaced to produce a gradation of effects.

(ii) The high dose level in rodents should elicit some signs of toxicity without causing excessive lethality; for non-rodents, there should be signs of toxicity but there should be no fatalities.

(iii) The lowest dose level should not produce any evidence of toxicity. Where there is a usable estimation of human exposure the lowest dose level should exceed this even though this dose level may result in some signs of toxicity.

(iv) Ideally, the intermediate dose level(s) should produce minimal observable toxic effects. If more than one intermediate dose is used, the dose level should be spaced to produce a gradation of toxic effects.

(v) For rodents, the incidence of fatalities in low and intermediate dose groups and in the controls should be low to permit a meaningful evaluation of the results. For non-rodents, there should be no fatalities.

(4) *Exposure conditions.* The animals are dosed with the test substance ideally on a 7-day per week basis over a period of at least 12 months. However, based primarily on practical considerations, dosing on a 5-day per week basis is considered to be acceptable.

(5) *Observation period.* Duration of observation should be for at least 12 months, and may be concurrent with or subsequent to dosing. If there is a post-exposure observation period, an interim sacrifice should be performed on no fewer than half of the animals of each sex at each dose level immediately upon termination of exposure.

(6) *Administration of the test substance.* The three main routes of administration are oral, dermal, and inhalation. The choice of the route of administration depends upon the physical and chemical characteristics

of the test substance and the form typifying exposure in humans.

(i) *Oral studies.* (A) The animals should receive the test substance in their diet, dissolved in drinking water, or given by gavage or capsule for a period of at least 12 months.

(B) If the test substance is administered in the drinking water, or mixed in the diet, exposure is continuous.

(C) For a diet mixture, the highest concentration should not exceed 5 percent.

(ii) *Dermal studies.* (A) The animals are treated by topical application with the test substance, ideally for at least 6 hours per day.

(B) Fur should be clipped from the dorsal area of the trunk of the test animals. Care must be taken to avoid abrading the skin which could alter its permeability.

(C) The test substance should be applied uniformly over a shaved area which is approximately 10 percent of the total body surface area. With highly toxic substances, the surface area covered may be less, but as much of the area should be covered with as thin and uniform a film as possible.

(D) During the exposure period, the test substance may be held if necessary, in contact with the skin with a porous gauze dressing and non-irritating tape. The test site should be further covered in a suitable manner to retain the gauze dressing and test substance and ensure that the animals cannot ingest the test substance.

(iii) *Inhalation studies.* (A) The animals should be tested with inhalation equipment designed to sustain a dynamic air flow of 12 to 15 air changes per hour, ensure an adequate oxygen content of 19 percent and an evenly distributed exposure atmosphere. Where a chamber is used, its design should minimize crowding of the test animals and maximize their exposure to the test substance. This is best accomplished by individual caging. As a general rule to ensure stability of a chamber atmosphere, the total "volume" of the test animals should not exceed 5 percent of the volume of the test chamber. Alternatively, oronasal, head-only or whole body individual chamber exposure may be used.

(B) The temperature at which the test is performed should be maintained at 22°C (±2°). Ideally, the relative humidity should be maintained between 40 to 60 percent, but in certain instances (e.g., tests of aerosols, use of water vehicle) this may not be practicable.

(C) Feed and water should be withheld during each daily 6 hour exposure period.

(D) A dynamic inhalation system with a suitable analytical concentration control system should be used. The rate of air flow should be adjusted to ensure that conditions throughout the equipment are essentially the same. Maintenance of slight negative pressure inside the chamber will prevent leakage of the test substance into the surrounding areas.

(7) *Observation of animals.* (i) Each animal should be handled and its physical condition appraised at least once each day.

(ii) Additional observations should be made daily with appropriate actions taken to minimize loss of animals to the study (e.g., necropsy or refrigeration of those animals found dead and isolation or sacrific of weak or moribund animals).

(iii) Clinical signs of toxicity including suspected tumors and mortality should be recorded as they are observed, including the time of onset, the degree and duration.

(iv) Cage-side observations should include, but not be limited to, changes in skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behavior pattern.

(v) Body weights should be recorded individually for all animals once a week during the first 13 weeks of the test period and at least once every 4 weeks thereafter unless signs of clinical toxicity suggest more frequent weighings to facilitate monitoring of health status.

(vi) When the test substance is administered in the feed or drinking water, measurements of feed or water consumption, respectively, should be determined weekly during the first 13 weeks of the study and then at approximately monthly intervals unless health status or body weight changes dictate otherwise.

(vii) At the end of the study period all survivors should be sacrificed. Moribund animals should be removed and sacrificed when noticed.

(8) *Physical measurements.* For inhalation studies, measurements or monitoring should be made of the following:

(i) The rate of air flow should be monitored continuously, but should be recorded at intervals of at least once every 30 minutes.

(ii) During each exposure period the actual concentrations of the test substance should be held as constant as practicable, monitored continuously and measured at least three times during the test period: at the beginning, at an intermediate time and at the end of the period.

(iii) During the development of the generating system, particle size analysis should be performed to establish the stability of aerosol concentrations. During exposure, analysis should be conducted as often as necessary to determine the consistency of particle size distribution and homogeneity of the exposure stream.

(iv) Temperature and humidity should be monitored continuously, but should be recorded at intervals of at least once every 30 minutes.

(9) *Clinical examinations.* The following examinations should be made on at least 10 rats of each sex per dose and on all non-rodents.

(i) Certain hematology determinations (e.g., hemoglobin content, packed cell volume, total red blood cells, total white blood cells, platelets, or other measures of clotting potential) should be performed at termination and should be performed at 3 months, 6 months and at approximately 6 month intervals thereafter (for studies extending beyond 12 months) on blood samples collected from all non-rodents and from 10 rats per sex of all groups. These collections should be from the same animals at each interval. If clinical observations suggest a deterioration in health of the animals during the study, a differential blood count of the affected animals should be performed. A differential blood count should be performed on samples from those animals in the highest dosage group and the controls. Differential blood counts should be performed for the next lower group(s) if there is a major discrepancy between the highest group and the controls. If hematological effects were noted in the subchronic test, hematological testing should be performed at 3, 6, 12, 18, and 24 months for a two year study and at 3, 6, and 12 months for a 1-year study.

(ii) Certain clinical biochemistry determinations on blood should be carried out at least three times during the test period: just prior to initiation of dosing (base line data), near the middle and at the end of the test period. Blood samples should be drawn for clinical chemistry measurements from all non-rodents and at least ten rodents per sex of all groups; if possible, from the same rodents at each time interval. Test areas which are considered appropriate to all studies: electrolyte balance, carbohydrate metabolism and liver and kidney function. The selection of specific tests will be influenced by observations on the mode of action of the substance and signs of clinical toxicity. Suggested chemical determinations: calcium, phosphorus, chloride, sodium, potassium, fasting glucose (with period of fasting appropriate to the species), serum glutamic-pyruvic transaminase (now known as serum alanine aminotransferase), serum glutamic oxaloacetic transaminase (now known as serum aspartate aminotransferase), ornithine decarboxylase, gamma glutamyl transpeptidase, blood urea nitrogen, albumen, blood creatinine, creatinine phosphokinase, total cholesterol, total bilirubin and total serum protein measurements. Other determinations which may be necessary for an adequate toxicological evaluation include analyses of lipids, hormones, acid/base balance, methemoglobin and cholinesterase activity. Additional clinical biochemistry may be employed where necessary to extend the investigation of observed effects.

(iii) Urine samples from rodents at the same intervals as the hematological examinations under paragraph (b)(9)(i) of this section should be collected for analysis. The following determinations should be made from either individual animals or on a pooled sample/sex/group for rodents: appearance (volume and specific gravity), protein, glucose, ketones, bilirubin, occult blood (semi-quantitatively); and microscopy of sediment (semi-quantitatively).

(iv) Ophthalmological examination, using an ophthalmoscope or equivalent suitable equipment, should be made prior to the administration of the test substance and at the termination of the study. If changes in eyes are detected all animals should be examined.

(10) *Gross necropsy.* (i) A complete gross examination should be performed on all animals, including those which died during the experiment or were killed in moribund conditions.

(ii) The liver, kidneys, adrenals, brain and gonads should be weighed wet, as soon as possible after dissection to avoid drying. For these organs, at least 10 rodents per sex per group and all non-rodents should be weighed.

(iii) The following organs and tissues, or representative samples thereof, should be preserved in a suitable medium for possible future histopathological examination: All gross lesions and tumors; brain—including sections of medulla/pons, cerebellar cortex, and cerebral cortex; pituitary; thyroid/parathyroid; thymus; lungs; trachea; heart; sternum and/or femur with bone marrow; salivary glands; liver; spleen; kidneys; adrenals; esophagus; stomach; duodenum; jejunum; ileum; cecum; colon; rectum; urinary bladder; representative lymph nodes; pancreas; gonads; uterus; accessory genital organs (epididymis, prostate, and, if present, seminal vesicles; female mammary gland; aorta; gall bladder (if present); skin; musculature; peripheral nerve; spinal cord at three levels—cervical, midthoracic, and lumbar; and eyes. In inhalation studies, the entire respiratory tract, including nose, pharynx, larynx, and paranasal sinuses should be examined and preserved. In dermal studies, skin from sites of skin painting should be examined and preserved.

(iv) Inflation of lungs and urinary bladder with a fixative is the optimal method for preservation of these tissues. The proper inflation and fixation of the lungs in inhalation studies is considered essential for appropriate and valid histopathological examination.

(v) If other clinical examinations are carried out, the information obtained from these procedures should be available before microscopic examination, since they may provide significant guidance to the pathologist.

(11) *Histopathology.* (i) The following histopathology should be performed:

(A) Full histopathology on the organs and tissues, listed above, of all non-rodents, of all rodents in the control and high dose groups and of all rodents that died or were killed during the study.

(B) All gross lesions in all animals.

(C) Target organs in all animals.

(D) Lungs, liver and kidneys of all animals. Special attention to examination of the lungs of rodents should be made for evidence of infection since this provides an assessment of the state of health of the animals.

(ii) If excessive early deaths or other problems occur in the high dose group compromising the significance of the data, the next dose level should be examined for complete histopathology.

(iii) In case the results of an experiment give evidence of substantial alteration of the animals' normal longevity or the induction of effects that might affect a toxic response, the next lower dose level should be examined fully, as described under paragraph (b)(11)(i) of this section.

(iv) An attempt should be made to correlate gross observations with microscopic findings.

(c) *Data and reporting*—(1) *Treatment of results.* (i) Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions, the types of lesions and the percentage of animals displaying each type of lesion.

(ii) All observed results, quantitative and incidental, should be evaluated by an appropriate statistical method. Any generally accepted statistical methods may be used; the statistical methods should be selected during the design of the study.

(2) *Evaluation of study results.* (i) The findings of a chronic toxicity study should be evaluated in conjunction with the findings of preceding studies and considered in terms of the toxic effects, the necropsy and histopathological findings. The evaluation will include the relationship between the dose of the test substance and the presence, incidence and severity of abnormalities (including behavioral and clinical abnormalities), gross lesions, identified target organs, body weight changes, effects on mortality and any other general or specific toxic effects.

(ii) In any study which demonstrates an absence of toxic effects, further investigation to establish absorption and bioavailability of the test substance should be considered.

(3) *Test report.* (i) In addition to the reporting requirements as specified under 40 CFR Part 792 Subpart J, the following specific information should be reported:

(A) *Group animal data.* Tabulation of toxic response data by species, strain, sex and exposure level for:

(*1*) Number of animals dying.

(*2*) Number of animals showing signs of toxicity.

(*3*) Number of animals exposed.

(B) *Individual animal data.* (*1*) Time of death during the study or whether animals survived to termination.

(*2*) Time of observation of each abnormal sign and its subsequent course.

(*3*) Body weight data.

(*4*) Feed and water consumption data, when collected.

(*5*) Results of ophthalmological examination, when performed.

(*6*) Hematological tests employed and all results.

(*7*) Clinical biochemistry tests employed and all results.

(*8*) Necropsy findings.

(*9*) Detailed description of all histopathological findings.

(*10*) Statistical treatment of results, where appropriate.

(ii) In addition, for inhalation studies the following should be reported:

(A) *Test conditions.* (*1*) Description of exposure apparatus including design, type, dimensions, source of air, system for generating particulates and aerosols, method of conditioning air, treatment of exhaust air and the method of housing the animals in a test chamber.

(*2*) The equipment for measuring temperature, humidity, and particulate aerosol concentrations and size should be described.

(B) *Exposure data.* These should be tabulated and presented with mean values and a measure of variability (e.g., standard deviation) and should include:

(*1*) Airflow rates through the inhalation equipment.

(*2*) Temperature and humidity of air.

(*3*) Nominal concentration (total amount of test substance fed into the inhalation equipment divided by volume of air).

(*4*) Actual concentration in test breathing zone.

(*5*) Particle size distribution (e.g., median aerodynamic diameter of particles with standard deviation from the mean).

(d) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Benitz, K.F. "Measurement of Chronic Toxicity," *Methods of Toxicology.* Ed. G.E. Paget. (Oxford: Blackwell Scientific Publications, 1970) pp. 82-131.

(2) D'Aguanno, W. "Drug Safety Evaluation—Pre-Clinical Considerations," *Industrial Pharmacology: Neuroleptics.* Vol. I, Ed. S. Fielding and H. Lal. (Mt. Kisco: Futura Publishing Co. 1974) pp. 317-332.

(3) Fitzhugh, O.G. Third Printing: 1975. "Chronic Oral Toxicity," *Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics.* The Association of Food and Drug Officials of the United States (1959, 3rd Printing 1975) pp. 36-45.

(4) Goldenthal, E.I., D'Aguanno, W. "Evaluation of Drugs," *Appraisal of the Safety of Chemicals in Foods, Drugs, and Cosmetics.* The Association of Food and Drug Officials of the United States (1959, 3rd Printing 1975) pp. 60-67.

(5) National Academy of Sciences. "Principles and Procedures for Evalu-

ating the Toxicity of Household Substances," a report prepared by the Committee for the Revision of NAS Publication 1138, under the auspices of the Committee on Toxicology, National Research Council, National Academy of Sciences, Washington, DC (1977).

(6) National Center for Toxicological Research. "Appendix B," *Report of Chronic Studies Task Force Committee, April 13-21, 1972.* (Rockville: National Center for Toxicological Research, 1972).

(7) Page, N.P. "Chronic Toxicity and Carcinogenicity Guidelines," *Journal of Environmental Pathology and Toxicology,* 1:161-182 (1977).

(8) Schwartz, E. "Toxicology of Neuroleptic Agents," *Industrial Pharmacology: Neuroleptics* Ed. S. Fielding and H. Lal. (Mt. Kisco, Futura Publishing Co., 1974) pp. 203-221.

(9) United States Pharmaceutical Manufacturers Association. *Guidelines for the Assessment of Drug and Medical Device Safety in Animals.* (1977).

(10) World Health Organization. "Guidelines for Evaluation of Drugs for Use in Man," *WHO Technical Report Series No. 563.* (Geneva: World Health Organization, 1975).

(11) World Health Organization. "Part I. Environmental Health Criteria 6," *Principles and Methods for Evaluating the Toxicity of Chemicals.* (Geneva: World Health Organization, 1978).

(12) World Health Organization. "Principles for Pre-Clinical Testing of Drug Safety," *WHO Technical Report Series No. 341.* (Geneva: World Health Organization, 1966).

[50 FR 39397, Sept. 27, 1985, as amended at 54 FR 21064, May 16, 1989]

## § 798.3300 Oncogenicity.

(a) *Purpose.* The objective of a long-term oncogenicity study is to observe test animals for a major portion of their life span for the development of neoplastic lesions during or after exposure to various doses of a test substance by an appropriate route of administration.

(b) *Test procedures*—(1) *Animal selection*—(i) *Species and strain.* A compound of unknown activity shall be tested on two mammalian species. Rats and mice are the species of choice because of their relatively short life spans, the limited cost of their maintenance, their widespread use in pharmacological and toxicological studies, their susceptibility to tumor induction, and the availability of inbred or sufficiently characterized strains. Commonly used laboratory strains shall be employed. If other species are used, the tester shall provide justification/reasoning for their selection.

(ii) *Age.* (A) Dosing of rodents shall begin as soon as possible after weaning, ideally before the animals are 6 weeks old, but in no case more than 8 weeks old.

(B) At commencement of the study, the weight variation of animals used shall not exceed ±20 percent of the mean weight for each sex.

(C) Studies using prenatal or neonatal animals may be recommended under special conditions.

(iii) *Sex.* (A) Animals of each sex shall be used at each dose level.

(B) The females shall be nulliparous and non-pregnant.

(iv) *Numbers.* (A) For rodents, at least 100 animals (50 females and 50 males) shall be used at each dose level and concurrent control.

(B) If interim sacrifices are planned the number shall be increased by the number of animals scheduled to be sacrificed during the course of the study.

(C) The number of animals at the termination of the study should be adequate for a meaningful and valid statistical evaluation of long term exposure. For a valid interpretation of negative results, it is essential that survival in all groups does not fall below 50 percent at the time of termination.

(2) *Control groups.* (i) A concurrent control group is required. This group shall be an untreated or sham treated control group or, if a vehicle is used in administering the test substance, a vehicle control group. If the toxic properties of the vehicle are not known or cannot be made available, both untreated and vehicle control groups are required.

(ii) In special circumstances such as in inhalation studies involving aerosols or the use of an emulsifier of uncharacterized biological activity in oral studies, a concurrent negative control group shall be utilized. The negative control group shall be treated in the same manner as all other test animals except that this control group shall not be exposed to either the test substance or any vehicle.

(iii) The use of historical control data (i.e., the incidence of tumors and other suspect lesions normally occurring under the same laboratory conditions and in the same strain of animals employed in the test) is desirable for assessing the significance of changes observed in exposed animals.

(3) *Dose levels and dose selection.* (i) For risk assessment purposes, at least 3 dose levels shall be used, in addition to the concurrent control group. Dose levels should be spaced to produce a gradation of chronic effects.

(ii) The high dose level should elicit signs of minimal toxicity without substantially altering the normal life span.

(iii) The lowest dose should not interfere with normal growth, development and longevity of the animal; and it should not otherwise cause any indication of toxicity. In general, this should not be lower than ten percent of the high dose.

(iv) The intermediate dose(s) should be established in a mid-range between the high and low doses, depending upon the toxicokinetic properties of the chemical, if known.

(v) The selection of these dose levels should be based on existing data, preferably on the results of subchronic studies.

(4) *Exposure conditions.* The animals are dosed with the test substance ideally on a 7 day per week basis over a period of at least 24 months for rats, and 18 months for mice. However, based primarily on practical considerations, dosing on a 5 day per week basis is considered to be acceptable.

(5) *Observations period.* It is necessary that the duration of an oncogenicity test comprise the majority of the normal life span of the strain of animals to be used. This time period shall not be less than 24 months for rats and 18 months for mice, and ordinarily not longer than 30 months for rats and 24 months for mice. For longer time periods, and where any other species are used, consultation with the Agency in regard to the duration of the test is advised.

(6) *Administration of the test substance.* The three main routes of administration are oral, dermal, and inhalation. The choice of the route of administration depends upon the physical and chemical characteristics of the test substance and the form typifying exposure in humans.

(i) *Oral studies.* (A) The animals shall receive the test substance in their diet, dissolved in drinking water at levels that do not exceed the maximum solubility of the test chemical under testing condition.

(B) If the test substance is administered in the drinking water, or mixed in the diet, exposure shall be continuous.

(C) For a diet mixture, the highest concentration should not exceed 5 percent.

(ii) *Dermal studies.* (A) The animals are treated by topical application with the test substance, ideally for at least 6 hours per day.

(B) Fur should be clipped from the dorsal area of the trunk of the test animals. Care should be taken to avoid abrading the skin which could alter its permeability.

(C) The test substance shall be applied uniformly over a shaved area which is approximately 10 percent of the total body surface area. With highly toxic substances, the surface area covered may be less, but as much of the area shall be covered with as thin and uniform a film as possible.

(D) During the exposure period, the test substance may be held, if necessary, in contact with the skin with a porous gauze dressing and non-irritating tape. The test site should be further covered in a suitable manner to retain the gauze dressing and test substance and ensure that the animals cannot ingest the test substance.

(iii) *Inhalation studies.* (A) The animals shall be tested with inhalation equipment designed to sustain a minimum dynamic air flow of 12 to 15 air changes per hour, ensure an adequate oxygen content of 19 percent and an evenly distributed exposure atmosphere. Where a chamber is used, its design should minimize crowding of the test animals and maximize their exposure to the test substance. This is best accomplished by individual caging. To ensure stability of a chamber atmosphere, the total "volume" of the test animals shall not exceed 5 percent of the volume of the test chamber. Alternatively, oro-nasal, head-only, or whole-body individual chamber exposure may be used.

(B) The temperature at which the test is performed should be maintained at 22 °C (±2°). Ideally, the relative humidity should be maintained between 40 to 60 percent, but in certain instances (e.g. tests of aerosols, use of water vehicle) this may not be practicable.

(C) Feed and water shall be withheld during each daily 6-hour exposure period.

(D) A dynamic inhalation system with a suitable flow control system shall be used. The rate of air flow shall be adjusted to ensure that conditions throughout the equipment are essentially the same. Maintenance of slight negative pressure inside the chamber will prevent leakage of the test substance into the surrounding areas.

(7) *Observations of animals.* (i) Each animal shall be observed daily and if necessary should be handled to appraise its physical condition.

(ii) Additional observations shall be made daily with appropriate actions taken to minimize loss of animals to the study (e.g., necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals).

(iii) Clinical signs and mortality shall be recorded for all animals. Special attention should be paid to tumor development. The day of onset, location, dimensions, appearance and progression of each grossly visible or palpable tumor shall be recorded.

(iv) Body weights shall be recorded individually for all animals once a week during the first 13 weeks of the test period and at least once every 4 weeks thereafter unless signs of clinical toxicity suggest more frequent weighings to facilitate monitoring of health status.

(v) When the test substance is administered in the feed or drinking water, measurements of feed or water consumption, respectively, shall be determined weekly during the first 13 weeks of the study and then at approximately monthly intervals unless health status or body weight changes dictate otherwise.

(vi) At the end of the study period all survivors are sacrificed. Moribund animals shall be removed and sacrificed when noticed.

(8) *Physical measurements.* For inhalation studies, measurements or monitoring should be made of the following:

(i) The rate of air flow shall be monitored continuously and recorded at intervals of at least once every 30 minutes.

(ii) During each exposure period the actual concentrations of the test substance shall be held as constant as practicable, monitored continuously and recorded at least three times during the test period: at the beginning, at an intermediate time and at the end of the period.

(iii) During the development of the generating system, particle size analysis shall be performed to establish the stability of aerosol concentrations with respect to particle size. During exposure, analyses shall be conducted as often as necessary to determine the consistency of particle size, distribution, and homogeneity of the exposure stream.

(iv) Temperature and humidity shall be monitored continuously, but shoud be recorded at intervals of at least once every 30 minutes.

(9) *Clinical examinations.* At 12 months, 18 months, and at sacrifice, a blood smear shall be obtained from all animals. A differential blood count shall be performed on blood smears from those animals in the highest dosage group and the controls. If these data, or data from the pathological examination indicate a need, then the 12- and 18-month blood smears from other dose levels shall also be examined. Differential blood counts shall be performed for the next lower group(s) if there is a major discrepancy between the highest group and the controls. If clinical observations sug-

gest a deterioration in health of the animals during the study, a differential blood count of the affected animals shall be performed.

(10) *Gross necropsy.* (i) A complete gross examination shall be performed on all animals, including those which died during the experiment or were killed in moribund conditions.

(ii) The following organs and tissues or representative samples thereof, shall be preserved in a suitable medium for possible future histopathological examination: All gross lesions and tumors of all animals shall be preserved; brain—including sections of medulla/pons, cerebellar cortex and cerebral cortex; pituitary; thyroid/parathyroid; thymus; lungs; trachea; heart; spinal cord at three levels—cervical, midthoracic and lumbar; sternum and/or femur with bone marrow; salivary glands; liver; spleen; kidneys; adrenals; esophagus; stomach; duodenum; jejunum; ileum; cecum; colon; rectum; urinary bladder; representative lymph nodes; pancreas; gonads; uterus; accessory genital organs (epididymis, prostate, and, if present, seminal vesicles); mammary gland; skin; musculature; peripheral nerve; and eyes. In inhalation studies, the entire respiratory tract shall be preserved, including nasal cavity, pharynx, larynx and paranasal sinuses. In dermal studies, skin from sites of skin painting shall be examined and preserved.

(iii) Inflation of lungs and urinary bladder with a fixative is the optimal method for preservation of these tissues. The proper inflation and fixation of the lungs in inhalation studies is required for appropriate and valid histopathological examination.

(iv) If other clinical examinations are carried out, the information obtained from these procedures shall be available before microscopic examination, since they may provide significant guidance to the pathologist.

(11) *Histopathology.* (i) The following histopathology shall be performed:

(A) Full histopathology on organs and tissues listed above of all animals in the control and high dose groups and all animals that died or were killed during the study.

(B) All gross lesions in all animals.

(C) Target organs in all animals.

(ii) If a significant difference is observed in hyperplastic, pre-neoplastic or neoplastic lesions between the highest dose and control groups, microscopic examination shall be made on that particular organ or tissue of all animals in the study.

(iii) If excessive early deaths or other problems occur in the high dose group, compromising the significance of the data, the next lower dose level shall be examined for complete histopathology.

(iv) In case the results of an experiment give evidence of substantial alteration of the animals' normal longevity or the induction of effects that might affect a neoplastic response, the next lower dose level shall be examined fully as described in this section.

(v) An attempt shall be made to correlate gross observations with microscopic findings.

(c) *Data and reporting*—(1) *Treatment of results.* (i) Data shall be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions, the types of lesions and the percentage of animals displaying each type of lesion.

(ii) All observed results, quantitative and incidental, shall be evaluated by an appropriate statistical method. Any generally accepted statistical method may be used; the statistical methods shall be selected during the design of the study.

(2) *Evaluation of study results.* (i) The findings of an oncogenic toxicity study shall be evaluated in conjunction with the findings of preceding studies and considered in terms of the toxic effects, the necropsy and histopathological findings. The evaluation shall include the relationship between the dose of the test substance and the presence, incidence and severity of abnormalities (including behavioral and clinical abnormalities), gross lesions, identified target organs, body weight changes, effects on mortality and any other general or specific toxic effects.

(ii) In any study which demonstrates an absence of toxic effects, further investigation to establish absorption and bioavailability of the test substance should be considered.

(iii) In order for a negative test to be acceptable, it shall meet the following criteria: no more than 10 percent of any group is lost due to autolysis, cannibalism, or management problems; and survival in each group should be no less than 50 percent at 18 months for mice and hamsters and at 24 months for rats.

(3) *Test report.* (i) In addition to the reporting requirements as specified under 40 CFR Part 792, Subpart J the following specific information shall be reported:

(A) *Group animal data.* Tabulation of toxic response data by species, strain, sex and exposure level for:

(*1*) Number of animals dying.

(*2*) Number of animals showing signs of toxicity.

(*3*) Number of animals exposed.

(B) *Individual animal data.* (*1*) Time of death during the study or whether animals survived to termination.

(*2*) Time of observation of each abnormal sign and its subsequent course.

(*3*) Body weight data.

(*4*) Feed and water consumption data, when collected.

(*5*) Results of ophthalmological examination, when performed.

(*6*) Hematological tests employed and all results.

(*7*) Clinical biochemistry tests employed and all results.

(*8*) Necropsy findings.

(*9*) Detailed description of all histopathological findings.

(*10*) Statistical treatment of results, where appropriate.

(*11*) Historical control data, if taken into account.

(ii) In addition, for inhalation studies the following shall be reported:

(A) *Test conditions.* (*1*) Description of exposure apparatus including design, type, dimensions, source of air, system for generating particulates and aerosols, method of conditioning air, treatment of exhaust air and the method of housing the animals in a test chamber.

(*2*) The equipment for measuring temperature, humidity, and particulate aerosol concentrations and size shall be described.

(B) *Exposure data.* These shall be tabulated and presented with mean values and a measure of variability (e.g., standard deviation) and shall include:

(*1*) Airflow rates through the inhalation equipment.

(*2*) Temperature and humidity of air.

(*3*) Nominal concentration (total amount of test substance fed into the inhalation equipment divided by volume of air).

(*4*) Actual concentration in test breathing zone.

(*5*) Particle size distribution (e.g., median aerodynamic diameter of particles with standard deviation from the mean).

(d) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Department of Health and Welfare. *The Testing of Chemicals for Carcinogenicity, Mutagenicity, Teratogenicity.* Minister of Health and Welfare. (Canada: Department of Health and Welfare, 1975).

(2) Food and Drug Administration Advisory Committee on Protocols for Safety Evaluation: Panel on Carcinogenesis. "Report on Cancer Testing in the Safety of Food Additives and Pesticides," *Toxicology and Applied Pharmacology.* 20:419-438 (1971).

(3) International Union Against Cancer. "Carcinogenicity Testing," *IUCC Technical Report Series.* Vol. 2., Ed. I. Berenblum. (Geneva: International Union Against Cancer, 1969).

(4) Leong, B.K.J., Laskin, S. "Number and Species of Experimental Animals for Inhalation Carcinogenicity Studies" Paper presented at Conference on Target Organ Toxicity, September 1975, Cincinnati, Ohio.

(5) National Academy of Sciences. "Principles and Procedures for Evaluating the Toxicity of Household Substances." A report prepared by the Committee for the Revision of NAS Publication 1138, under the auspices of the Committee on Toxicology, National Research Council, National Academy of Sciences, Washington, DC (1977).

(6) National Cancer Institute. *Report of the Subtask Group on Carcinogen Testing to the Interagency Collaborative Group on Environmental Car-*

*cinogenesis*. (Bethesda: United States National Cancer Institute, 1976).

(7) National Center for Toxicological Research. "Appendix B," *Report of Chronic Studies Task Force Committee*. April 13-21 (Rockville: National Center for Toxicological Research, 1972).

(8) Page, N.P. "Chronic Toxicity and Carcinogenicity Guidelines," *Journal of Environmental Pathology and Toxicology*. 1:161-182 (1977).

(9) Page, N.P. "Concepts of a Bioassay Program in Environmental Carcinogenesis," *Advances in Modern Toxicology Vol. 3*, Ed. Kraybill and Mehlman. (Washington, DC: Hemisphere Publishing Corporation, 1977) pp. 87-171.

(10) Sontag, J.M., Page N.P., Saffiotti, U. *Guidelines for Carcinogen Bioassay in Small Rodents*. NCI-CS-TR-1. (Bethesda: United States Cancer Institute, Division of Cancer Control and Prevention, Carcinogenesis Bioassay Program, 1976).

(11) United States Pharmaceutical Manufacturers Association. *Guidelines for the Assessment of Drug and Medical Device Safety in Animals*. (1977).

(12) World Health Organization. "Principles for the Testing and Evaluation of Drugs for Carcinogenicity," *WHO Technical Report Series No. 426*. (Geneva: World Health Organization, 1969).

(13) World Health Organization. "Part I. Environmental Health Criteria 6," *Principles and Methods for Evaluating the Toxicity of Chemicals*. (Geneva: World Health Organization, 1978).

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### § 798.3320 Combined chronic toxicity/oncogenicity.

(a) *Purpose*. The objective of a combined chronic toxicity/oncogenicity study is to determine the effects of a substance in a mammalian species following prolonged and repeated exposure. The application of this guideline should generate data which identify the majority of chronic and oncogenic effects and determine dose-response relationships. The design and conduct should allow for the detection of neoplastic effects and a determination of oncogenic potential as well as general toxicity, including neurological, physiological, biochemical, and hematological effects and exposure-related morphological (pathology) effects.

(b) *Test procedures*—(1) *Animal selection*—(i) *Species and strain*. Preliminary studies providing data on acute, subchronic, and metabolic responses should have been carried out to permit an appropriate choice of animals (species and strain). As discussed in other guidelines, the mouse and rat have been most widely used for assessment of oncogenic potential, while the rat and dog have been most often studied for chronic toxicity. The rat is the species of choice for combined chronic toxicity and oncogenicity studies. The provisions of this guideline are designed primarily for use with the rat as the test species. If other species are used, the tester should provide justification/reasoning for their selection. The strain selected should be susceptible to the oncogenic or toxic effect of the class of substances being tested, if known, and provided it does not have a spontaneous background too high for meaningful assessment. Commonly used laboratory strains should be employed.

(ii) *Age*. (A) Dosing of rats should begin as soon as possible after weaning, ideally before the rats are 6 weeks old, but in no case more than 8 weeks old.

(B) At commencement of the study, the weight variation of animals used should not exceed ±20 percent of the mean weight for each sex.

(C) Studies using prenatal or neonatal animals may be recommended under special conditions.

(iii) *Sex*. (A) Equal numbers of animals of each sex should be used at each dose level.

(B) The females should be nulliparous and nonpregnant.

(iv) *Numbers*. (A) At least 100 rodents (50 females and 50 males) should be used at each dose level and concurrent control for those groups not intended for early sacrifice. At least 40 rodents (20 females and 20 males) should be used for satellite dose group(s) and the satellite control group. The purpose of the satellite group is to allow for the evaluation of pathology other than neoplasia.

(B) If interim sacrifices are planned, the number of animals should be increased by the number of animals scheduled to be sacrificed during the course of the study.

(C) The number of animals at the termination of each phase of the study should be adequate for a meaningful and valid statistical evaluation of long term exposure. For a valid interpretation of negative results, it is essential that survival in all groups not fall below 50 percent at the time of termination.

(2) *Control groups.* (i) A concurrent control group (50 females and 50 males) and a satellite control group (20 females and 20 males) are recommended. These groups should be untreated or sham treated control groups or, if a vehicle is used in administering the test substance, vehicle control groups. If the toxic properties of the vehicle are not known or cannot be made available, both untreated and vehicle control groups are recommended. Animals in the satellite control group should be sacrificed at the same time the satellite test group is terminated.

(ii) In special circumstances such as inhalation studies involving aerosols or the use of an emulsifier of uncharacterized biological activity in oral studies, a concurrent negative control group should be utilized. The negative control group should be treated in the same manner as all other test animals, except that this control group should not be exposed to the test substance or any vehicle.

(iii) The use of historical control data (i.e., the incidence of tumors and other suspect lesions normally occuring under the same laboratory conditions and in the same strain of animals employed in the test) is desirable for assessing the significance of changes observed in exposed animals.

(3) *Dose levels and dose selection.* (i) For risk assessment purposes, at least three dose levels should be used, in addition to the concurrent control group. Dose levels should be spaced to produce a gradation of effects.

(ii) The highest dose level in rodents should elicit signs of toxicity without substantially altering the normal life span due to effects other than tumors.

(iii) The lowest dose level should produce no evidence of toxicity. Where there is a usable estimation of human exposure, the lowest dose level should exceed this even though this dose level may result in some signs of toxicity.

(iv) Ideally, the intermediate dose level(s) should produce minimal observable toxic effects. If more than one intermediate dose is used the dose levels should be spaced to produce a gradation of toxic effects.

(v) For rodents, the incidence of fatalities in low and intermediate dose groups and in the controls should be low to permit a meaningful evaluation of the results.

(vi) For chronic toxicological assessment, a high dose treated satellite and a concurrent control satellite group should be included in the study design. The highest dose for satellite animals should be chosen so as to produce frank toxicity, but not excessive lethality, in order to elucidate a chronic toxicological profile of the test substance. If more than one dose level is selected for satellite dose groups, the doses should be spaced to produce a gradation of toxic effects.

(4) *Exposure conditions.* The animals are dosed with the test substance ideally on a 7-day per week basis over a period of at least 24 months for rats, and 18 months for mice and hamsters, except for the animals in the satellite groups which should be dosed for 12 months.

(5) *Observation period.* It is necessary that the duration of the oncogenicity test comprise the majority of the normal life span of the animals to be used. It has been suggested that the duration of the study should be for the entire lifetime of all animals. However, a few animals may greatly exceed the average lifetime and the duration of the study may be unnecessarily extended and complicate the conduct and evaluation of the study. Rather, a finite period covering the majority of the expected life span of the strain is preferred since the probability is high that, for the great majority of chemicals, induced tumors will occur within

such an observation period. The following guidelines are recommended:

(i) Generally, the termination of the study should be at 18 months for mice and hamsters and 24 months for rats; however, for certain strains of animals with greater longevity and/or low spontaneous tumor rate, termination should be at 24 months for mice and hamsters and at 30 months for rats. For longer time periods, and where any other species are used, consultation with the Agency in regard to duration of the test is advised.

(ii) However, termination of the study is acceptable when the number of survivors of the lower doses or of the control group reaches 25 percent. In the case where only the high dose group dies prematurely for obvious reasons of toxicity, this should not trigger termination of the study.

(iii) The satellite groups and the concurrent satellite control group should be retained in the study for at least 12 months. These groups should be scheduled for sacrifice for an estimation of test-substance-related pathology uncomplicated by geriatric changes.

(6) *Administration of the test substance.* The three main routes of administration are oral, dermal, and inhalation. The choice of the route of administration depends upon the physical and chemical characteristics of the test substance and the form typifying exposure in humans.

(i) *Oral studies.* (A) The animals should receive the test substance in their diet, dissolved in drinking water, or given by gavage or capsule for a period of at least 24 months for rats and 18 months for mice and hamsters.

(B) If the test substance is administered in the drinking water, or mixed in the diet, exposure is continuous.

(C) For a diet mixture, the highest concentration should not exceed 5 percent.

(ii) *Dermal studies.* (A) The animals are treated by topical application with the test substance, ideally for at least 6 hours per day.

(B) Fur should be clipped from the dorsal area of the trunk of the test animals. Care should be taken to avoid abrading the skin which could alter its permeability.

(C) The test substance should be applied uniformly over a shaved area which is approximately 10 percent of the total body surface area. With highly toxic substances, the surface area covered may be less, but as much of the area as possible should be covered with as thin and uniform a film as possible.

(D) During the exposure period, the test substance may be held, if necessary, in contact with the skin with a porous gauze dressing and nonirritating tape. The test site should be further covered in a suitable manner to retain the gauze dressing and test substance and ensure that the animals cannot ingest the test substance.

(iii) *Inhalation studies.* (A) The animals should be tested with inhalation equipment designed to sustain a dynamic air flow of 12 to 15 air changes per hour, to ensure an adequate oxygen content of 19 percent and an evenly distributed exposure atmosphere. Where a chamber is used, its design should minimize crowding of the test animals and maximize their exposure to the test substance. This is best accomplished by individual caging. As a general rule, to ensure stability of a chamber atmosphere, the total "volume" of the test animals should not exceed 5 percent of the volume of the test chamber. Alternatively, oro-nasal, head only, or whole body individual chamber exposure may be used.

(B) The temperature at which the test is performed should be maintained at 22 °C (±2°). Ideally, the relative humidity should be maintained between 40 to 60 percent, but in certain instances (e.g., tests of aerosols, use of water vehicle) this may not be practicable.

(C) Feed and water should be withheld during each daily 6-hour exposure period.

(D) A dynamic inhalation system with a suitable analytical concentration control system should be used. The rate of air flow should be adjusted to ensure that conditions throughout the equipment are essentially the same. Maintenance of slight negative pressure inside the chamber will prevent leakage of the test substance into the surrounding areas.

(7) *Observation of animals.* (i) Each animal should be handled and its physical condition appraised at least once each day.

(ii) Additional observations should be made daily with appropriate actions taken to minimize loss of animals to the study (e.g., necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals).

(iii) Clinical signs and mortality should be recorded for all animals. Special attention should be paid to tumor development. The time of onset, location, dimensions, appearance and progression of each grossly visible or palpable tumor should be recorded.

(iv) Body weights should be recorded individually for all animals once a week during the first 13 weeks of the test period and at least once every 4 weeks thereafter, unless signs of clinical toxicity suggest more frequent weighings to facilitate monitoring of health status.

(v) When the test substance is administered in the feed or drinking water, measurements of feed or water consumption, respectively, should be determined weekly during the first 13 weeks of the study and then at approximately monthly intervals unless health status or body weight changes dictate otherwise.

(vi) At the end of the study period, all survivors are sacrificed. Moribund animals should be removed and sacrificed when noticed.

(8) *Physical measurements.* For inhalation studies, measurements or monitoring should be made of the following:

(i) The rate of airflow should be monitored continuously, but should be recorded at intervals of at least once every 30 minutes.

(ii) During each exposure period the actual concentrations of the test substance should be held as constant as practicable, monitored continuously and recorded at least three times during the test period: At the beginning, at an intermediate time and at the end of the period.

(iii) During the development of the generating system, particle size analysis should be performed to establish the stability of aerosol concentrations. During exposure, analyses should be conducted as often as necessary to determine the consistency of particle size distribution and homogeneity of the exposure stream.

(iv) Temperature and humidity should be monitored continuously, but should be recorded at intervals of at least once every 30 minutes.

(9) *Clinical examinations.* (i) The following examinations should be made on at least 20 rodents of each sex per dose level:

(A) Certain hematology determinations (e.g., hemoglobin content, packed cell volume, total red blood cells, total white blood cells, platelets, or other measures of clotting potential) should be performed at termination and should be performed at 3 months, 6 months and at approximately 6-month intervals thereafter (for those groups on test for longer than 12 months) on blood samples collected from 20 rodents per sex of all groups. These collections should be from the same animals at each interval. If clinical observations suggest a deterioration in health of the animals during the study, a differential blood count of the affected animals should be performed. A differential blood count should be performed on samples from animals in the highest dosage group and the controls. Differential blood counts should be performed for the next lower group(s) if there is a major discrepancy between the highest group and the controls. If hematological effects were noted in the subchronic test, hematological testing should be performed at 3, 6, 12, 18 and 24 months for a year study.

(B) Certain clinical biochemistry determinations on blood should be carried out at least three times during the test period: Just prior to initiation of dosing (baseline data), near the middle and at the end of the test period. Blood samples should be drawn for clinical measurements from at least ten rodents per sex of all groups; if possible, from the same rodents at each time interval. Test areas which are considered appropriate to all studies: electrolyte balance, carbohydrate metabolism and liver and kidney function. The selection of specific tests will be influenced by observations on the mode of action of the substance and signs of clinical toxicity. Suggested chemical determinations: Calcium, phosphorus, chloride, sodium, potassium, fasting glucose (with period of fasting appropriate to the species), serum glutamic-pyruvic transaminase (now known as serum alanine aminotransferase), serum glutamic oxaloacetic transaminase (now known as serum aspartate aminotransferase), ornithine decarboxylase, gamma glutamyl transpeptidase, blood urea nitrogen, albumen, creatinine phosphokinase, total cholesterol, total bilirubin and total serum protein measurements. Other determinations which may be necessary for an adequate toxicological evaluation include analyses of lipids, hormones, acid/base balance, methemoglobin and cholinesterase activity. Additional clinical biochemistry may be employed where necessary to extend the investigation of observed effects.

(ii) The following should be performed on at least 10 rodents of each sex per dose level:

(A) Urine samples from the same rodents at the same intervals as hematological examination above, should be collected for analysis. The following determinations should be made from either individual animals or on a pooled sample/sex/group for rodents: appearance (volume and specific gravity), protein, glucose, ketones, bilirubin, occult blood (semi-quantitatively) and microscopy of sediment (semiquantitatively).

(B) Ophthalmological examination, using an ophthalmoscope or equivalent suitable equipment, should be made prior to the administration of the test substance and at the termination of the study. If changes in the eyes are detected, all animals should be examined.

(10) *Gross necropsy.* (i) A complete gross examination should be performed on all animals, including those which died during the experiment or were killed in moribund conditions.

(ii) The liver, kidneys, adrenals, brain and gonads should be weighed wet, as soon as possible after dissection to avoid drying. For these organs, at least 10 rodents per sex per group should be weighed.

(iii) The following organs and tissues, or representative samples thereof, should be preserved in a suitable medium for possible future histopathological examination: All gross lesions and tumors; brain-including sections of medulla/pons, cerebellar cortex, and cerebral cortex; pituitary; thyroid/parathyroid; thymus; lungs; trachea; heart; sternum and/or femur with bone marrow; salivary glands; liver; spleen; kidneys; adrenals; esophagus; stomach; duodenum; jejunum; ileum; cecum; colon; rectum; urinary bladder; representative lymph nodes; pancreas; gonads; uterus; accessory genital organs (epididymis, prostate, and, if present, seminal vesicles); female mammary gland; aorta; gall bladder (if present); skin; musculature; peripheral nerve; spinal cord at three levels—cervical, midthoracic, and lumbar; and eyes. In inhalation studies, the entire respiratory tract, including nose, pharynx, larynx and paranasal sinuses should be examined and preserved. In dermal studies, skin from sites of skin painting should be examined and preserved.

(iv) Inflation of lungs and urinary bladder with a fixative is the optimal method for preservation of these tissues. The proper inflation and fixation of the lungs in inhalation studies is considered essential for appropriate and valid histopathological examination.

(v) If other clinical examinations are carried out, the information obtained from these procedures should be available before microscopic examination, since they may provide significant guidance to the pathologist.

(11) *Histopathology.* (i) The following histopathology should be performed:

(A) Full histopathology on the organs and tissues, listed above, of all non-rodents, of all rodents in the control and high dose groups and of all rodents that died or were killed during the study.

(B) All gross lesions in all animals.

(C) Target organs in all animals.

(D) Lungs, liver and kidneys of all animals. Special attention to examination of the lungs of rodents should be made for evidence of infection since this provides an assessment of the state of health of the animals.

(ii) If excessive early deaths or other problems occur in the high dose group compromising the significance of the data, the next dose level should be examined for complete histopathology.

(iii) In case the results of the experiment give evidence of substantial alteration of the animals' normal longevity or the induction of effects that might affect a toxic response, the next lower dose level should be examined as described above.

(iv) An attempt should be made to correlate gross observations with microscopic findings.

(c) *Data and reporting*—(1) *Treatment of results.* (i) Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions, the types of lesions and the percentage of animals displaying each type of lesion.

(ii) All observed results, quantitative and incidental, should be evaluated by an appropriate statistical method. Any generally accepted statistical methods may be used; the statistical methods should be selected during the design of the study.

(2) *Evaluation of study results.* (i) The findings of a combined chronic toxicity/oncogenicity study should be evaluated in conjunction with the findings of preceding studies and considered in terms of the toxic effects, the necropsy and histopathological findings. The evaluation will include the relationship between the dose of the test substance and the presence, incidence and severity of abnormalities (including behavioral and clinical abnormalities), gross lesions, identified target organs, body weight changes, effects on mortality and any other general or specific toxic effects.

(ii) In any study which demonstrates an absence of toxic effects, further investigation to establish absorption and bioavailability of the test substance should be considered.

(iii) In order for a negative test to be acceptable, it should meet the following criteria: No more than 10 percent of any group is lost due to autolysis, cannibalism, or management problems; and survival in each group is no less than 50 percent at 18 months for mice and hamsters and at 24 months for rats.

(3) *Test report.* (i) In addition to the reporting requirements as specified under 40 CFR Part 792, Subpart J the following specific information should be reported:

(A) *Group animal data.* Tabulation of toxic response data by species, strain, sex and exposure level for:

(*1*) Number of animals dying.

(*2*) Number of animals showing signs of toxicity.

(*3*) Number of animals exposed.

(B) *Individual animal data.* (*1*) Time of death during the study or whether animals survived to termination.

(*2*) Time of observation of each abnormal sign and its subsequent course.

(*3*) Body weight data.

(*4*) Feed and water consumption data, when collected.

(*5*) Results of ophthalmological examination, when performed.

(*6*) Hematological tests employed and all results.

(*7*) Clinical biochemistry tests employed and all results.

(*8*) Necropsy findings.

(*9*) Detailed description of all histopathological findings.

(*10*) Statistical treatment of results where appropriate.

(*11*) Historical control data, if taken into account.

(ii) In addition, for inhalation studies the following should be reported:

(A) *Test Conditions.* (*1*) Description of exposure apparatus including design, type, dimensions, source of air, system for generating particulates and aerosols, method of conditioning air, treatment of exhaust air and the method of housing the animals in a test chamber.

(*2*) The equipment for measuring temperature, humidity, and particulate aerosol concentrations and size should be described.

(B) *Exposure data.* These should be tabulated and presented with mean values and a measure of variability (e.g. standard deviation) and should include:

(*1*) Airflow rates through the inhalation equipment.

(*2*) Temperature and humidity of air.

(*3*) Nominal concentration (total amount of test substance fed into the inhalation equipment divided by volume of air).

(*4*) Actual concentration in test breathing zone.

(*5*) Particle size distribution (e.g. median aerodynamic diameter of particles with standard deviation from the mean).

(d) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Benitz, K.F. "Measurement of Chronic Toxicity," *Methods of Toxicology.* Ed. G.E. Paget. (Oxford: Blackwell Scientific Publications, 1970) pp. 82–131.

(2) D'Aguanno, W. "Drug Safety Evaluation—Pre-Clinical Considerations," "*Industrial Pharmacology: Neuroleptics.* Vol. I Ed. S. Fielding and H. Lal. (Mt. Kisco, New York: Futura Publishing Co., 1974) pp. 317–332.

(3) Department of Health and Welfare. *The Testing of Chemicals for Carcinogenicity, Mutagenicity, Teratogenicity. Minister of Health and Welfare. (Canada: Department of Health and Welfare, 1975).*

(4) Fitzhugh, O.G. "Chronic Oral Toxicity," *Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics.* The Association of Food and Drug Officials of the United States (1959, 3rd Printing 1975). pp. 36–45.

(5) Food and Drug Administration Advisory Committee on Protocols for Safety Evaluation: Panel on Carcinogenesis. "*Report on Cancer Testing in the Safety of Food Additives and Pesticides,*" *Toxicology and Applied Pharmacology.* 20:419-438 (1971).

(6) Goldenthal, E.I., and D'Aguanno, W. "Evaluation of Drugs," *Appraisal of the Safety of Chemicals in Foods, Drugs, and Cosmetics.* The Association of Food and Drug Officials of the United States (1959, 3rd printing 1975) pp.60–67.

(7) International Union Against Cancer. "Carcinogenicity Testing," *IUCC Technical Report Series* Vol. 2, Ed. I. Berenblum. (Geneva: International Union Against Cancer, 1969).

(8) Leong, B.K.J., and Laskin, S. "Number and Species of Experimental Animals for Inhalation Carcinogenicity Studies," Paper presented at Conference on Target Organ Toxicity. September, 1975, Cincinnati, Ohio.

(9) National Academy of Sciences. "Principles and Procedures for Evaluating the Toxicity of Household Substances," A report prepared by the Committee for the Revision of NAS Publication 1138, under the auspices of the Committee on Toxicology, National Research Council, National Academy of Sciences, Washington, DC (1977).

(10) National Cancer Institute. *Report of the Subtask Group on Carcinogen Testing to the Interagency Collaborative Group on Environmental Carcinogenesis.* (Bethesda: United States National Cancer Institute, 1976).

(11) National Center for Toxicological. *Report of Chronic Studies Task Force Research Committee. "Appendix B, (Rockville: National Center for Toxicological Research, 1972)).*

(12) Page, N.P. "Chronic Toxicity and Carcinogenicity Guidelines," *Journal Environmental Pathology and Toxicology.* 1:161–182 (1977).

(13) Page, N.P. "Concepts of a Bioassay Program in Environmental Carcinogenesis," *Advances in Modern Toxicology* Volume 3, Ed. Kraybill and Mehlman. (Washington, D.C.: Hemisphere Publishing Corp., 1977) pp. 87–171.

(14) Schwartz, E. 1974. "Toxicology of Neuroleptic Agents," *Industrial Pharmacology: Neuroleptics.* Ed. S. Fielding and H. Lal. (Mt. Kisco, New York: Futura Publishing Co, 1974) pp. 203–221.

(15) Sontag, J.M., Page, N.P., and Saffiotti, U. *Guidelines for Carcinogen Bioassay in Small Rodents.* NCI-CS-TR-1 (Bethesda: United States Cancer Institute, Division of Cancer Control and Prevention, Carcinogenesis Bioassay Program, 1976).

(16) United States Pharmaceutical Manufacturers Association. *Guidelines for the Assessment of Drug and Medical Device Safety in Animals.* (1977).

(17) World Health Organization. "Principles for the Testing and Evaluation of Drugs for Carcinogenicity," *WHO Technical Report Series No. 426.* (Geneva: World Health Organization, 1969).

(18) World Health Organization. "Guidelines for Evaluation of Drugs for Use in Man," *WHO Technical Report Series No. 563.* (Geneva: World Health Organization, 1975).

(19) World Health Organization. "Part I. Environmental Health Criteria 6," *Principles and Methods for Evaluating the Toxicity of Chemicals.* (Geneva: World Health Organization, 1978).

(20) World Health Organization. "Principles for Pre-Clinical Testing of Drug Safety," *WHO Technical Report Series No. 341.* (Geneva: World Health Organization, 1966).

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## Subpart E—Specific Organ/Tissue Toxicity

### § 798.4100 Dermal sensitization.

(a) *Purpose.* In the assessment and evaluation of the toxic characteristics of a substance, determination of its potential to provoke skin sensitization reactions is important. Information derived from tests for skin sensitization serves to identify the possible hazard to a population repeatedly exposed to a test substance. While the desirability of skin sensitization testing is recognized, there are some real differences of opinion about the best method to use. The test selected should be a reliable screening procedure which should not fail to identify substances with significant allergenic potential, while at the same time avoiding false negative results.

(b) *Definitions.* (1) Skin sensitization (allergic contact dermatitis) is an immunologically mediated cutaneous reaction to a substance. In the human, the responses may be characterized by pruritis, erythema, edema, papules, vesicles, bullae, or a combination of these. In other species the reactions may differ and only erythema and edema may be seen.

(2) Induction period is a period of at least 1 week following a sensitization exposure during which a hypersensitive state is developed.

(3) Induction exposure is an experimental exposure of a subject to a test substance with the intention of inducing a hypersensitive state.

(4) Challenge exposure is an experimental exposure of a previously treated subject to a test substance following an induction period, to determine whether the subject will react in a hypersensitive manner.

(c) *Principle of the test method.* Following initial exposure(s) to a test substance, the animals are subsequently subjected, after a period of not less than 1 week, to a challenge exposure with the test substance to establish whether a hypersensitive state has been induced. Sensitization is determined by examining the reaction to the challenge exposure and comparing this reaction to that of the initial induction exposure.

(d) *Test procedures.* (1) Any of the following seven test methods is considered to be acceptable. It is realized, however, that the methods differ in their probability and degree of reaction to sensitizing substances.

(i) Freund's complete adjuvant test.

(ii) Guinea-pig maximization test.

(iii) Split adjuvant technique.

(iv) Buehler test.

(v) Open epicutaneous test.

(vi) Mauer optimization test.

(vii) Footpad technique in guinea pig.

(2) Removal of hair is by clipping, shaving, or possibly by depilation, depending on the test method used.

(3) *Animal selection*—(i) *Species and strain.* The young adult guinea pig is the preferred species. Commonly used laboratory strains should be employed. If other species are used, the tester should provide justification/reasoning for their selection.

(ii) *Number and sex.* (A) The number and sex of animals used will depend on the method employed.

(B) The females should be nulliparous and nonpregnant.

(4) *Control animals.* (i) Periodic use of a positive control substance with an acceptable level of reliability for the test system selected is recommended;

(ii) Animals may act as their own controls or groups of induced animals can be compared to groups which have received only a challenge exposure.

(5) *Dose levels.* The dose level will depend upon the method selected.

(6) *Observation of animals.* (i) Skin reactions should be graded and recorded after the challenge exposures at the time specified by the methodology selected. This is usually at 24, 48, and 72, hours. Additional notations should be made as necessary to fully describe unusual responses;

(ii) Regardless of method selected, initial and terminal body weights should be recorded.

(7) *Procedures.* The procedures to be used are those described by the methodology chosen.

(e) *Data and reporting.* (1) Data should be summarized in tabular form, showing for each individual animal the skin reaction, results of the induction exposure(s) and the challenge exposure(s) at times indicated by the method chosen. As a minimum, the erythema and edema should be graded and any unusual finding should be recorded.

(2) *Evaluation of the results.* The evaluation of results will provide information on the proportion of each group that became sensitized and the extent (slight, moderate, severe) of the sensitization reaction in each individual animal.

(3) *Test report.* In addition to the reporting requirements as specified under 40 CFR Part 792, Subpart J, the following specific information should be reported:

(i) A description of the method used and the commonly accepted name.

(ii) Information on the positive control study, including positive control used, method used, and time conducted.

(iii) The number and sex of the test animals.

(iv) Species and strain.

(v) Individual weights of the animals at the start of the test and at the conclusion of the test.

(vi) A brief description of the grading system.

(vii) Each reading made on each individual animal.

(f) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Buehler, E.V. "Delayed Contact Hypersensitivity in the Guinea Pig," *Archives Dermatology.* 91:171 (1965).

(2) Draize, J.H. "Dermal Toxicity," *Food Drug Cosmetic Law Journal.* 10:722-732 (1955).

(3) Klecak, G. "Identification of Contact Allergens: Predictive Tests in Animals," *Advances in Modern Toxicology: Dermatology and Pharmacology.* Ed. F.N. Marzulli and H.I. Maibach. (Washington, D.C.: Hemisphere Publishing Corp., 1977) 4:305-339).

(4) Klecak, G., Geleick, H., Grey, J.R. "Screening of Fragrance Materials for Allergenicity in the Guinea Pig.-1. Comparison of Four Testing Methods," *Journal of the Society of Cosmetic Chemists.* 28:53-64 (1977).

(5) Magnusson, B., Kligman, A.M. "The Identification of Contact Allergens by Animal Assay," The Guinea Pig Maximization Test. *The Journal of Investigative Dermatology.* 52:268-276 (1973).

(6) Maguire, H.C. "The Bioassay of Contact Allergens in the Guinea Pig" *Journal of the Society of Cosmetic Chemists.* 24:151-162 (1973).

(7) Maurer, T., Thomann, P., Weirich, E.G., Hess, R. "The Optimization Test in the Guinea Pig. A Method for the Predictive Evaluation of the Contact Allergenicity of Chemicals," *Agents and Actions.* (Basel: Birkhauser Verlag, 1975) Vol. 5/2.

(8) Maurer, T., Thomann, P., Weirich, E.G., Hess, R. "The Optimization Test in the Guinea Pig: A Method for the Predictive Evaluation of the Contact Allergenicity of Chemicals," *International Congress Series Excerpta Medica No. 376,* (1975) Vol. 203.

## § 798.4350 Inhalation developmental toxicity study.

(a) *Purpose.* In the assessment and evaluation of the toxic characteristics of an inhalable material such as a gas, volatile substance, or aerosol/particulate, determination of the potential developmental toxicity is important. The inhalation developmental toxicity study is designed to provide information on the potential hazard to the unborn which may arise from exposure of the mother during pregnancy.

(b) *Definitions.* (1) Developmental toxicity is the property of a chemical that causes in utero death, structural or functional abnormalities or growth retardation during the period of development.

(2) "Aerodynamic diameter" applies to the behavioral size of particles of aerosols. It is the diameter of a sphere of unit density which behaves aerodynamically like the particles of the test substance. It is used to compare particles of different sizes, shapes, and densities and to predict where in the respiratory tract such particles may be deposited. This term is used in contrast to "optical," "measured" or "geometric" diameters which are representation of actual diameters which in themselves cannot be related to deposition within the respiratory tract.

(3) "Geometric mean diameter" or "median diameter" is the calculated aerodynamic diameter which divides the particles of an aerosol in half based on the weight of the particles. Fifty percent of the particles by weight will be larger than the median diameter and 50 percent of the particles will be smaller than the median diameter. The median diameter and its geometeric standard deviation are used to statistically describe the particle size distribution of any aerosol based on the weight and size of the particles.

(4) "Inhalable diameter" refers to that aerodynamic diameter of a particle which is considered to be inhalable for the organism. It is used to refer to particles which are capable of being inhaled and may be deposited anywhere within the respiratory tract from the trachea to the deep lung (the alveoli). For man, the inhalable diameter is considered here as 15 micrometers or less.

(5) "Concentration" refers to an exposure level. Exposure is expressed as weight or volume of test substance per volume of air (mg/1), or as parts per million (ppm).

(6) "No-observed-effect level" is the maximum concentration in a test which produces no observed adverse effects. A no-observed-effect level is expressed in terms of weight or volume of test substance given daily per unit volume of air.

(c) *Principle of the test method.* The test substance is administered in graduated concentrations, for at least that part of the pregnancy covering the major period of organogenesis, to several groups of pregnant experimental animals, one exposure level being used per group. Shortly before the expected date of delivery, the pregnant females are sacrificed, the uteri removed, and the contents examined for embryonic or fetal deaths, and live fetuses.

(d) *Limit test.* If a test at an exposure of 5 mg/1 (actual concentration of respirable substances) or, where this is not possible due to physical or chemical properties of the test substance, the maximum attainable concentration, produces no observable developmental toxicity, then a full study using three exposure levels might not be necessary.

(e) *Test procedures*— (1) *Animal selection*—(i) *Species and strain.* Testing shall be performed in at least two mamalian species. Commonly used species include the rat, mouse, rabbit, and hamster. If other mamalian species are used, the tester shall provide justification/reasoning for their selection. Commonly used laboratory strains shall be employed. The strain shall not have low fecundity and shall preferably be characterized for its sensitivity to developmental toxins.

(ii) *Age.* Young adult animals (nulliparous females) shall be used.

(iii) *Sex.* Pregnant female animals shall be used at each exposure level.

(iv) *Number of animals.* At least 20 pregnant rats, mice, or hamsters or 12 pregnant rabbits are required at each exposure level. The objective is to ensure that sufficient pups are produced to permit meaningful evaluation of the potential developmental toxicity of the test substance.

(2) *Control group.* A concurrent control group shall be used. This group shall be exposed to clean, filtered air under conditions identical to those used for the group exposed to the substance of interest. In addition, a vehicle-exposed group may be necessary when the substance under study requires a vehicle for delivery. It is rec-

ommended that during preliminary range finding studies, air vs. vehicle exposure be compared. If there is no substantial difference, air exposure itself would be an appropriate control. If vehicle and air exposure yield different results, both vehicle and air exposed control groups are recommended.

(3) *Concentration levels and concentration selection.* (i) At least three concentration levels with a control and, where appropriate, a vehicle control, shall be used.

(ii) The vehicle shall neither be developmentally toxic nor have effects on reproduction.

(iii) To select the appropriate concentration levels, a pilot or trial study may be advisable. Since pregnant animals have an increased minute ventilation as compared to non-pregnant animals, it is recommended that the trial study be conducted in pregnant animals. Similarly, since presumably the minute ventilation will vary with progression of pregnancy, the animals should be exposed during the same period of gestation as in the main study. In the trial study, the concentration producing embryonic or fetal lethalities or maternal toxicity should be determined.

(iv) Unless limited by the physical/chemical nature or biological properties of the substance, the highest concentration level shall induce some overt maternal toxicity such as reduced body weight or body weight gain, but not more than 10 percent maternal deaths.

(v) The lowest concentration level should not produce any grossly observable evidence of either maternal or developmental toxicity.

(vi) Ideally, the intermediate concentration level(s) shall produce minimal observable toxic effects. If more than one intermediate concentration is used, the concentration levels shall be spaced to produce a gradation of toxic effects.

(4) *Exposure duration.* The duration of exposure shall be at least six hours daily allowing appropriate additional time for chamber equilibrium.

(5) *Observation period.* Day 0 in the test is the day on which a vaginal plug and/or sperm are observed. The exposure period shall cover the period of major organogenesis. This may be taken as days 6 to 15 for rat and mouse, 6 to 14 for hamster, or 6 to 18 for rabbit.

(6) *Inhalation exposure.* (i)(A) The animals shall be tested in inhalation equipment designed to sustain a minimum dynamic air flow of 12 to 15 air changes per hour and ensure an adequate oxygen content of 19 percent and an evenly distributed exposure atmosphere. Where a chamber is used, its design should minimize crowding of the test animals and maximize their exposure to the test substance. This is best accomplished by individual caging. To ensure stability of a chamber atmosphere, the total "volume" of the test animals shall not exceed 5 percent of the volume of the test chamber.

(B) Pregnant animals shall not be subjected to beyond the minimum amount of stress. Since whole-body exposure appears to be the least stressful mode of exposure, it is the method preferred. In general oro-nasal or head-only exposure, which is sometimes used to avoid concurrent exposure by the dermal or oral routes, is not recommended because of the associated stress accompanying the restraining of the animals. However, there may be specific instances where it may be more appropriate than whole-body exposure. The tester shall provide justification/reasoning for its selection.

(ii) A dynamic inhalation system with a suitable flow control system shall be used. The rate of air flow shall be adjusted to ensure that conditions throughout the exposure chamber are essentially the same. Test material distribution should be established before animals are committed to dosing. Maintenance of slight negative pressure inside the chamber will prevent leakage of the test substance into the surrounding areas.

(iii) The temperature at which the test is performed should be maintained at 22 °C (±2°) for rodents or 20 °C (±3°) for rabbits. Ideally, the relative humidity should be maintained between 40 to 60 percent, but in certain instances (e.g., tests of aerosols, use of water vehicle) this may not be practicable.

(7) *Physical measurements.* Measurements or monitoring should be made of the following:

(i) The rate of airflow shall be monitored continuously but shall be recorded at least every 30 minutes.

(ii) The actual concentration of the test substance shall be measured in the breathing zone. During the exposure period the actual concentrations of the test substance shall be held as constant as practicable, monitored continously or intermittently depending on the method of analysis and measured at least at the beginning, at an intermediate time and at the end of the exposure period.

(iii) During the development of the generating system, particle size analysis shall be performed to establish the stability of aerosol concentrations with respect to particle size. During exposure, analysis shall be conducted as often as necessary to determine the consistency of particle size distribution.

(iv) Temperature and humidity shall be monitored continuously and be recorded at least every 30 minutes.

(8) *Food and water during exposure period.* Food should be withheld during exposure. Water may or may not be withheld. If it is not withheld it should not come in direct contact with the test atmospheres.

(9) *Observation of animals.* (i) A gross examination shall be made at least once each day.

(ii) Additional observations should be made daily with appropriate actions taken to minimize loss of animals to the study (e.g., necropsy or refrigeration of animals found dead and isolation or sacrifice of weak or moribund animals).

(iii) Signs of toxicity shall be recorded as they are observed, including the time of onset, the degree and duration.

(iv) Cage-side observations shall include, but not be limited to: Changes in skin and fur, eye and mucous membranes, as well as respiratory, autonomic and central nervous systems, somatomotor activity and behavioral pattern. Particular attention should be directed to observation of tremors, convulsions, salivation, diarrhea, lethargy, sleep, and coma.

(v) Measurements should be made weekly of food consumption for all animals in the study.

(vi) Animals shall be weighed at least weekly.

(vii) Females showing signs of abortion or premature delivery shall be sacrificed and subjected to a thorough macroscopic examination.

(10) *Gross necropsy.* (i) At the time of sacrifice or death during the study, the dam shall be examined macroscopically for any structural abnormalities or pathological changes which may have influenced the pregnancy.

(ii) Immediately after sacrifice or death, the uterus shall be removed, weighed, and the contents examined for embryonic or fetal deaths and the number of viable fetuses. Gravid uterine weights should not be obtained from dead animals if autolysis or where decomposition has occurred. The degree of resorption shall be described in order to help estimate the relative time of death.

(iii) The number of corpora lutea shall be determined for all species except mice.

(iv) The sex of the fetuses shall be determined and they shall be weighed individually, the weights recorded, and the mean fetal weight derived.

(v) Following removal, each fetus shall be examined externally.

(vi) For rats, mice and hamsters, one-third to one-half of each litter shall be prepared and examined for skeletal anomalies, and the remaining part of each litter shall be prepared and examined for soft tissue anomalies using appropriate methods.

(vii) For rabbits, each fetus shall be examined by careful dissection for visceral anomalies and then examined for skeletal anomalies.

(f) *Data and reporting*—(1) *Treatment of results.* Data shall be summarized in tabular form, showing for each test group: the number of animals at the start of the test, the number of pregnant animals, the number and percentages of live fetuses and the number of fetuses with any soft tissue or skeletal abnormalities.

(2) *Evaluation of results.* The findings of a developmental toxicity study

shall be evaluated in terms of the observed effects and the exposure levels producing effects. It is necessary to consider the historical developmental toxicity data on the species/strain tested. A properly conducted developmental toxicity study should provide a satisfactory estimation of a no-effect level.

(3) *Test report.* In addition to the reporting requirements as specified under 40 CFR Part 792, Subpart J, the following specific information shall be reported:

(i) *Test conditions.* (A) Description of exposure apparatus including design, type, dimensions, source of air, system for generating particulates and aerosols, methods of conditioning air, and the method of housing the animals in a test chamber when this apparatus is used.

(B) The equipment for measuring temperature, humidity, and particulate aerosol concentrations and size shall be described.

(ii) *Exposure data.* These shall be tabulated and presented with mean values and a measure of variability (e.g., standard deviation) and shall include:

(A) Airflow rates through the inhalation equipment.

(B) Temperature of air.

(C) Nominal concentration—total amount of test substance fed into the inhalation equipment divided by volume of air (no standard deviation).

(D) Measured total concentrations (particulate and/or gaseous phases) in test breathing zone.

(E) Particle size distribution (e.g., median aerodynamic diameter of particles with geometric standard deviation) including estimates of the percents of inhalable and non-inhalable portions for the test animals.

(iii) *Animal data.* (A) Toxic response data by concentration.

(B) Species and strain.

(C) Date of death during the study or whether animals survived to termination.

(D) Date of onset and duration of each abnormal sign and its subsequent course.

(E) Feed, body weight and uterine weight data.

(F) Pregnancy and litter data.

(G) Fetal data (live/dead, sex, soft tissue and sketetal defects, resorptions).

(g) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Department of Health and Welfare. *The Testing of Chemicals for Carcinogenicity, Mutagenicity and Teratogenicity.* Minister of Health and Welfare (Canada: Department of Health and Welfare, 1975).

(2) National Academy of Sciences. "Principles and Procedures for Evaluating the Toxicity of Household Substances." A report prepared by the Committee for the Revision of NAS Publication 1138, under the auspices of the Committee on Toxicology, National Research Council, National Academy of Sciences, Washington, DC (1977).

(3) World Health Organization. *Principles for the Testing of Drugs for Teratogenicity.* WHO Technical Report Series No. 364. (Geneva: World Health Organization, 1967).

[50 FR 39397, Sept. 27, 1985, as amended at 52 FR 19076, May 20, 1987; 52 FR 26150, July 13, 1987; 54 FR 21064, May 16, 1989]

### § 798.4420 Preliminary developmental toxicity screen.

(a) *Purpose.* The *in vivo* developmental toxicity assay is designed to assess the potential of agents to induce toxic effects in the conceptus after administration to the mother during pregnancy. This test should be used only to prioritize environmental agents for testing by more rigorous standard protocols.

(b) *Definitions.* Development toxicity is the capability of an agent to induce *in utero* death, structural or functional abnormalities, or growth retardation after contact with the pregnant animal.

(c) *Principle of the test method.* The test substance is administered to pregnant animals during a significant portion of the major period of organogenesis. A single dose level is administered. This dose level shall be high enough to elicit significant maternal toxicity. The dams are allowed to give birth and the neonates are counted and weighed on days 1 and 3 postpartum (day 1 is the day after birth). The underlying hypothesis for this assay is that most prenatal insults will manifest themselves as reduced viability and/or growth during the postnatal period.

(d) *Test procedures*—(1) *Animal selection*—(i) *Species and strain.* Testing must be performed in a mammalian species and strain which will allow human handling of newborn pups without cannibalization or abandonment. The preferred species would be either rat or mouse, and of necessity, a strain that does not exhibit the behavior referred to above. The strain should be commonly used and should not have low fecundity.

(ii) *Age.* Young adult animals (nulliparous females) shall be used.

(iii) *Sex.* Pregnant female animals shall be used.

(iv) *Number of animals.* At least 30 bred animals shall be used for each compound. The objective is to ensure that sufficient litters are produced to permit meaningful evaluation of the potential developmental toxicity of the test substance.

(2) *Control group.* A concurrent control group shall be used. This group shall be an untreated or sham treated control group, or, if a vehicle is used in administering the test substance, a vehicle control group. Except for treatment with the test substance, animals in the control group(s) shall be handled in an identical manner to test group animals.

(3) *Dose levels and dose selection.* (i) A single dose level with a concurrent control and, when appropriate, a vehicle control, should be used.

(ii) The vehicle shall be neither developmentally toxic nor have effects on reproduction.

(iii) To select the appropriate dose levels, a pilot or trial study may be advisable. It is not always necessary to carry out a trial study in pregnant animals. Comparison of the results from a trial study in non-pregnant, and the main study in pregnant animals will demonstrate whether or not the test substance is more toxic in pregnant animals.

(iv) Unless limited by the physical/chemical nature or biological properties of the substance, the dose level used shall be:

(A) High enough to cause overt maternal toxicity as evidenced by significant death, weight loss or other adverse clinical manifestations, or

(B) 1 gm/kg, if lower dose levels fail to induce maternal toxicity.

(4) *Observation period.* Day 0 in the test is the day in which a vaginal plug and/or sperm are observed. The dose period shall encompass a significant portion of the period of major organogenesis. This may be taken as days 7-11 for rat and mouse. When there is evidence of rapid clearance it may be advisable to extend the dosing period for 2 days to cover the critical period of palatal closure.

(5) *Administration of test substance.* The test substance or vehicle is usually administered orally, by intubation unless the chemical or physical characteristics of the test substance or pattern of human exposure suggest a more appropriate route of administration. The test substance shall be administered at approximately the same time each day.

(6) *Exposure conditions.* The female test animals are treated with the test substance daily throughout the appropriate treatment period. When given by gavage, the dose may be based on the weight of the females at the start of substance administration, or, alternatively, in view of the rapid weight gain which takes place during pregnancy, the animals may be weighed periodically and the dosage based on the most recent weight determination.

(7) *Observation of pregnant animals.* (i) A gross examination of the dams shall be made at least once prior to parturition.

(ii) Pregnant animals shall be weighed the day prior to the beginning of treatment, and that day on which treatment ends.

(iii) Cage-side observations shall include, but not be limited to: change in skin and fur, eye and mucous membranes, as well as respiratory, autonomic and central nervous systems, somatomotor activity and behavioral pattern.

(iv) Signs of toxicity shall be recorded as they are observed, including the time of onset, the degree and duration.

(v) During the dosing period females that die or are sacrificed because they are moribund shall be examined for signs of pregnancy and details of the conditions of the uterus and/or its contents recorded. Animals that have not delivered two days after expected date of parturition should be sacrificed and similar examinations made.

(8) *Observation of dams after birth.* Dams shall be observed for signs of overt toxicity during the postpartum period at the same time neonatal examinations are being made.

(9) *Neonatal examinations.* (i) Dams are allowed to give birth and the litters are examined for gross anomalies and presence of milk, counted, and weighed on postpartum days 1 and 3.

(ii) Dead pups shall be subjected to a thorough external examination and gross soft tissue abnormalities noted.

(iii) For those compounds that induce only neonatal growth reduction it may be advisable to normalize litter size on postpartum day 3 (to approximately 4 females and 4 males) and leave them with the dam through weaning. This procedure will determine if the growth reduction is transient or if it represents a permanent functional alteration.

(e) *Data and reporting*—(1) *Treatment of results.* Data shall be summarized in tabular form, showing for each test group: the number of animals at the start of the test, the number of pregnant animals, the maternal weight during the treatment period, the average number of live neonates on days 1 and 3, the average neonatal weight on days 1 and 3, and the average weight gained during that period.

(2) *Evaluation of results.* The findings of this bioassay shall be evaluated in terms of the types of effects noted. All data analyses shall compare treatment groups and their concurrent controls. Statistical treatment of the results shall involve analysis of variance, and the number of live pups on days 1 and 3 shall be used as a covariate in the analyses of postnatal body weight so as to correct for differences in pup weights due to litter size. The number of animals going to term must be sufficiently large to allow for a reasonable detection of compound-induced deficiencies. Conditions which significantly reduce the number of dams going to term (e.g. lack of pregnancy or compound-induced maternal death) shall lead to a repeat of the study.

(3) *Test report.* In addition to the reporting requirements as specified under 40 CFR Part 792, Subpart J the following specific information shall be reported:

(i) Toxic response data.

(ii) Species and strain.

(iii) Date of maternal death during the study or whether animals survived to termination.

(iv) Date of onset and duration of each abnormal sign and its subsequent course.

(v) Pregnancy data.

(vi) Litter data including number live and dead; and average litter weight on days 1 and 3 postpartum.

(vii) Necropsy data on dead pups.

(f) *References.* For additional background information on this test guideline the following references should be consulted.

(1) Chernoff, N. and Kavlock, R. "An *in vivo* teratology screen utilizing pregnant mice," *Journal of Toxicology and Environmental Health* 10: 541-550 (1982).

(2) Doe, J. E., Samuels, D. M., Tinston, D. J. and De Silva Wickramaratne, G. A. "Comparative aspects of the reproductive toxicology by inhalation in rats of ethylene glycol monomethyl ether and propylene glycol monomethyl ether," *Toxicology and Applied Pharmacology.* 69(1): 43-47 (1983).

[50 FR 39397, Sept. 27, 1985, as amended at 52 FR 19077, May 20, 1987]

### § 798.4470 Primary dermal irritation.

(a) *Purpose.* In the assessment and evaluation of the toxic characteristics of a substance, determination of the irritant and/or corrosive effects on skin of mammals is an important initial step. Information derived from this test serves to indicate the existence of possible hazards likely to arise from exposure of the skin to the test substance.

(b) *Definitions.* (1) "Dermal irritation" is the production of reversible inflammatory changes in the skin following the application of a test substance.

(2) "Dermal corrosion" is the production of irreversible tissue damage in the skin following the application of the test substance.

(c) *Principle of the test methods.* (1) The substance to be tested is applied in a single dose to the skin of several experimental animals, each animal serving as its own control. The degree of irritation is read and scored at specified intervals and is further described to provide a complete evaluation of the effects. The duration of the study should be sufficient to permit a full evaluation of the reversibility or irreversibility of the effects observed but need not exceed 14 days.

(2) When testing solids (which may be pulverized if considered necessary), the test substance should be moistened sufficiently with water or, where necessary, a suitable vehicle, to ensure good contact with the skin. When vehicles are used, the influence of the vehicle on irritation of skin by the test substance should be taken into account. Liquid test substances are generally used undiluted.

(3) Strongly acidic or alkaline substances, for example with a demonstrated pH of 2 or less, or 11.5 or greater, need not be tested for primary dermal irritation, owing to their predictable corrosive properties.

(4) The testing of materials which have been shown to be highly toxic by the dermal route is unnecessary.

(d) *Test procedures*—(1) *Animal selection*—(i) *Species and strain.* The albino rabbit is recommended as the preferred species. If another mammalian species is used, the tester should provide justification/reasoning for its selection.

(ii) *Number of animals.* At least 6 healthy adult animals should be used unless, justification/reasoning for using fewer animals is provided.

(2) *Control animals.* Separate animals are not recommended for an untreated control group. Adjacent areas of untreated skin of each animal may serve as a control for the test.

(3) *Dose level.* A dose of 0.5 ml of liquid or 500 mg of solid or semi-solid is applied to the test site.

(4) *Preparation of animals' skins.* Approximately 24 hours before the test, fur should be removed from the test area by clipping or shaving from the dorsal area of the trunk of the animals. Care should be taken to avoid abrading the skin. Only animals with healthy intact skin should be used.

(5) *Application of the test substance.* (i) The recommended exposure duration is 4-hours. Longer exposure may be indicated under certain conditions (e.g. expected pattern of human use and exposure). At the end of the exposure period, residual test substance should generally be removed, where practicable, using water or an appropriate solvent, without altering the existing response or the integrity of the epidermis.

(ii) The test substance should be applied to a small area (approximately 6 $cm^2$) of skin and covered with a gauze patch, which is held in place with nonirritating tape. In the case of liquids or some pastes, it may be necessary to apply the test substance to the gauze patch and then apply that to the skin. The patch should be loosely held in contact with the skin by means of a suitable semiocclusive dressing for the duration of the exposure period. However, the use of an occlusive dressing may be consided appropriate in some cases. Access by the animal to the patch and resultant ingestion/inhalation of the test substance should be prevented.

(6) *Observation period.* The duration of the observation period should be at least 72 hours, but should not be rigidly fixed. It should be sufficient to fully evaluate the reversibility or irreversibility of the effects observed. It need not exceed 14 days after application.

(7) *Clinical examination and scoring.* (i) After removal of the patch, animals should be examined for signs of erythema and edema and the responses scored within 30-60 minutes, and then at 24, 48, and 72 hours after patch removal.

(ii) Dermal irritation should be scored and recorded according to the grades in the following Table 1. Further observations may be needed, as necessary, to establish reversibility. In addition to the observation of irritation, any lesions and other toxic effects should be fully described.

TABLE 1—EVALUATION OF SKIN REACTION

| Formation | Value |
|---|---|
| Erythema and Eschar Formation: | |
| No erythema | 0 |
| Very slight erythema (barely perceptible) | 1 |
| Well-defined erythema | 2 |
| Moderate to severe erythema | 3 |
| Severe erythema (beet redness) to slight eschar formation (injuries in depth) | 4 |
| Maximum possible | 4 |
| Edema Formation: | |
| No edema | 0 |
| Very slight edema (barely perceptible) | 1 |
| Slight edema (edges of area well defined by definite raising) | 2 |
| Moderate edema (raised approximately 1 millimeter) | 3 |
| Severe edema (raised more than 1 millimeter and extending beyond area of exposure | 4 |
| Maximum possible | 4 |

(e) *Data and reporting*—(1) *Data summary.* Data should be summarized in tabular form, showing for each individual animal the irritation scores for erythema and edema at 30 to 60 minutes, 24, 48, and 72 hours after patch removal, any lesions, a description of the degree and nature of irritation, corrosion or reversibility, and any other toxic effects observed.

(2) *Evaluation of results.* The dermal irritation scores should be evaluated in conjunction with the nature and reversibility or otherwise of the responses observed. The individual scores do not represent an absolute standard for the irritant properties of a material. They should be viewed as reference values which are only meaningful when supported by a full description and evaluation of the observations. The use of an occlusive dressing is a severe test and the results are relevant to very few likely human exposure conditions.

(3) *Test report.* In addition to the reporting recommendations as specified under 40 CFR Part 792, Subpart J, the following specific information should be reported:

(i) Physical nature and where appropriate, concentration, and pH value for the test substance.

(ii) Species and strain.

(iii) Tabulation of irritation response data for each individual animal for each observation time period (e.g. 30 to 60 minutes, 24, 48, and 72 hours after patch removal).

(iv) Description of any lesions observed.

(v) Narrative description of the degree and nature of irritation observed.

(vi) Description of any toxic effects other than dermal irritation.

(f) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Draize, J.H. "Dermal Toxicity," *Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics.* Association of Food and Drug Officials of the United States (1959, 3rd printing 1975) pp. 46-59.

(2) Draize, J.H. Woodward, G., Calvery, H.O. "Methods for the Study of Irritation and Toxicity of Substances Applied Topically to the Skin and Mucous Membranes" *Journal of Pharmacology and Experimental Therapeutics.* 83:377-390 (1944).

(3) Marzulli, F.N., Maibach, H.I. "Dermatotoxicology and Pharmacology," *Advances in Modern Toxicology.* Vol. 4. (New York: Hemisphere Publishing Corp., 1977).

(4) National Academy of Sciences. "Principles and Procedures for Evaluating the Toxicity of Household Substances," A report prepared by the Committee for the Revision of NAS Publication 1138, Under the auspices of the Committee on Toxicology, National Research Council, National Academy of Sciences, Washington, DC, (1978).

(5) World Health Organization. "Part I. Environmental Health Criteria 6," *Principles and Methods for Evaluating the Toxicity of Chemicals.* (Geneva: World Health Organization, 1978).

## § 798.4500 Primary eye irritation.

(a) *Purpose.* In the assessment and evaluation of the toxic characteristics of a substance, determination of the irritant and/or corrosive effects on eyes of mammals is an important initial step. Information derived from this test serves to indicate the existence of possible hazards likely to arise from exposure of the eyes and associated mucous membranes to the test substance.

(b) *Definitions*—(1) Eye irritation. The production of reversible changes in the eye following the application of a test substance to the anterior surface of the eye.

(2) Eye corrosion. The production of irreversible tissue damage in the eye following application of a test substance to the anterior surface of the eye.

(c) Principle of the test method. (1) The substance to be tested is applied in a single dose to one of the eyes in each of several experimental animals; the untreated eye is used to provide control information. The degree of irritation/corrosion is evaluated and scored at specified intervals and is fully described to provide a complete evaluation of the effects. The duration of the study should be sufficient to permit a full evaluation of the reversibility or irreversibility of the effects observed but need not exceed 21 days.

(2) Strongly acidic or alkaline substances, for example, with a demonstrated pH of 2 or less, or 11.5 or greater, need not be tested owing to their predictable corrosive properties.

(3) Materials which have demonstrated definite corrosion or severe irritation in a dermal study need not be further tested for eye irritation. It may be presumed that such substances will produce similarly severe effects in the eyes.

(d) *Test procedures*—(1) *Animal selection*—(i) *Species and strain.* A variety of experimental animals have been used, but it is recommended that testing should be performed using healthy adult albino rabbits. Commonly used laboratory strains should be used. If another mammalian species is used, the tester should provide justification/reasoning for its selection.

(ii) *Number of animals.* At least six animals should be used, unless justification/reasoning for using fewer animals is provided.

(2) *Dose level.* For testing liquids, a dose of 0.1 ml is recommended. In testing solids, pastes, and particulate substances the amount used should have a volume of 0.1 ml, or a weight of not more than 100 mg (the weight must always be recorded). If the test material is solid or granular, it should be ground to a fine dust. The volume of particulates should be measured after gently compacting them (e.g. by tapping the measuring container). To test a substance contained in a pressurized aerosol container, the eye should be held open and the test substance administered in a single burst of about one second from a distance of 10 cm directly in front of the eye. The dose may be estimated by weighing the container before and after use. Care should be taken not to damage the eye. Pump sprays should not be used but instead the liquid should be expelled and 0.1 ml collected and instilled into the eye as described for liquids.

(3) *Examination of eyes prior to test.* Both eyes of each experimental animal provisionally selected for testing should be examined within 24 hours before testing starts by the same procedure to be used during the test examination. Animals showing eye irritation, ocular defects or preexisting corneal injury should not be used.

(4) *Application of the test substance.* (i) The test substance should be placed in the conjunctival sac of one eye of each animal after gently pulling the lower lid away from the eyeball. The lids are then gently held together for about one second in order to limit loss of the material. The other eye, which remains untreated, serves as a control. If it is thought that the substance may cause extreme pain, local anesthetic may be used prior to instillation of the test substance. The type and concentration of the local anesthetic should be carefully selected to ensure that no significant differences in reaction to the test substance will result from its use. The control eye should be similarly anesthetized.

(ii) The eyes of the test animals should not be washed out for 24 hours following instillation of the test substance. At 24 hours, a washout may be used if considered appropriate.

(5) *Observation period.* The duration of the observation period is at least 72 hours, but should not be fixed rigidly. It should be sufficient to evaluate fully the reversibility or irreversibility of the effects observed. It normally

need not exceed 21 days after instillation.

(6) *Clinical examination and scoring.* (i) The eyes should be examined at 1, 24, 48, and 72 hours. If there is no evidence of irritation at 72 hours, the study may be ended. Extended observation may be necessary if there is persistent corneal involvement or other ocular irritation in order to determine the progress of the lesions and their reversibility or irreversibility. In addition to the observations of the cornea, iris and conjunctivae, any other lesions which are noted should be recorded and reported. The grades of ocular reaction using the following Table I should be recorded at each examination.

*Table I—Grades for Ocular Lesions*

| | |
|---|---|
| **Cornea** | |
| Opacity: Degree of density (area most dense taken for reading). No ulceration or opacity | 0 |
| Scattered or diffuse areas of opacity (other than slight dulling of normal luster), details of iris clearly visible | [1]1 |
| Easily discernible translucent area, details of iris slightly obscured | [1]2 |
| Nacrous area, no details or iris visible, size of pupil barely discernible | [1]3 |
| Opaque cornea, iris not discernible through the opacity | [1]4 |
| **Iris** | |
| Normal | 0 |
| Markedly deepened rugae, congestion, swelling moderate circumcorneal hyperemia, or injection, any of these or combination of any thereof, iris still reacting to light (sluggish reaction is positive) | [1]1 |
| No reaction to light, hemorrhage, gross destruction (any or all of these) | [1]2 |
| **Conjunctivae** | |
| Redness (refers to palpebral and bulbar conjunctivae, cornea and iris). | |
| Blood vessels normal | 0 |
| Some blood vessels definitely hyperemic (injected) | 1 |
| Diffuse, crimson color, individual vessels not easily discernible | [1]2 |
| Diffuse beefy red chemosis: lids and/or nictitating membranes | [1]3 |
| No swelling | 0 |
| Any swelling above normal (includes nictitating membranes) | 1 |
| Obvious swelling with partial eversion of lids | [1]2 |
| Swelling with lids about half closed | [1]3 |
| Swelling with lids more than half closed | [1]4 |

[1] Indicates positive effect.

(ii) Examination of reactions can be facilitated by use of a binocular loupe, hand slit-lamp, biomicroscope, or other suitable device. After recording the observations at 24 hours, the eyes of any or all rabbits may be further examined with the aid of fluorescein.

(iii) The grading of ocular responses is subject to various interpretations. To promote harmonization and to assist testing laboratories and those involved in making and interpreting the observations, an illustrated guide in grading eye irritation should be used. (Such an illustrated guide is in use in the United States and can be obtained from the Consumer Product Safety Commission, Washington, DC 20207.)

(e) *Data and reporting*—(1) *Data summary.* Data should be summarized in tabular form, showing for each individual animal the irritation scores at the designated observation time; a description of the degree and nature of irritation; the presence of serious lesions and any effects other than ocular which were observed.

(2) *Evaluation of the results.* The ocular irritation scores should be evaluated in conjunction with the nature and reversibility or otherwise of the responses observed. The individual scores do not represent an absolute standard for the irritant properties of a material. They should be viewed as reference values and are only meaningful when supported by a full description and evaluation of the observations.

(3) *Test report.* In addition to the reporting requirements as specified under 40 CFR Part 792, Subpart J the following specific information should be reported:

(i) Physical nature and, where appropriate, concentration and pH value for the test substance.

(ii) Species and strain.

(iii) Tabulation of irritant/corrosive response data for each individual animal at each observation time point (e.g., 1, 24, 48, and 72 hours).

(iv) Description of any lesions observed.

(v) Narrative description of the degree and nature of irritation or corrosion observed.

(vi) Description of the method used to score the irritation at 1, 24, 48, and 72 hours (e.g., hand slit-lamp, biomicroscope, fluorescein).

(vii) Description of any non-ocular effects noted.

(f) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Buehler, EV., Newmann, E.A. "A Comparison of Eye Irritation in Monkeys and Rabbits," *Toxicology and Applied Pharmacology.* 6:701-710 (1964).

(2) Draize, J.H. "Dermal Toxicity," *Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics.* The Association of Food and Drug Officials of the United States (1959, 3rd printing 1975), pp. 49-52.

(3) Draize, J.H., Woodward, G., Calvery, H.O. "Methods for the Study of Irritation and Toxicity of Substances Applied Topically to the Skin and Mucous Membranes," *Journal of Pharmacology and Experimental Therapeutics.* 83:377-390 (1944).

(4) Loomis, T.A. *Essentials of Toxicology.* Second, (Philadelphia: Lea and Febicer, 1974), pp. 207-213.

(5) National Academy of Sciences. "Principles and Procedures for Evaluating the Toxicity of Household Substances." A report propared by the Committee for the revision of NAS Publication 1138, under the auspices of the Committee on Toxicology, National Research Council, National Academy of Sciences, Washington, DC (1977).

(6) World Health Organization. "Part I. Environmental Health Criteria 6," *Principles and Methods for Evaluating the Toxicity of Chemicals.* (Geneva: World Health Organization, 1978).

### § 798.4700 Reproduction and fertility effects.

(a) *Purpose.* This guideline for two-generation reproduction testing is designed to provide general information concerning the effects of a test substance on gonadal function, conception, parturition, and the growth and development of the offspring. The study may also provide information about the effects of the test substance on neonatal morbidity, mortality, and preliminary data on teratogenesis and serve as a guide for subsequent tests.

(b) *Principle of the test method.* The test substance is administered to parental (P) animals prior to their mating, during the resultant pregnancies, and through the weaning of their $F_1$ offspring. The substance is then administered to selected $F_1$ offspring during their growth into adulthood, mating, and production of an $F_2$ generation, up until the $F_2$ generation is weaned.

(c) *Test procedures*—(1) *Animal selection*—(i) *Species and strain.* The rat is the preferred species. If another mammalian species is used, the tester shall provide justification/reasoning for its selection. Strains with low fecundity shall not be used.

(ii) *Age.* Parental (P) animals shall be about 5 to 8 weeks old at the start of dosing.

(iii) *Sex.* (A) For an adequate assessment of fertility, both males and females shall be studied.

(B) The females shall be nulliparous and non-pregnant.

(iv) *Number of animals.* Each test and control group shall contain at least 20 males and a sufficient number of females to yield at least 20 pregnant females at or near term.

(2) *Control groups.* (i) A concurrent control group shall be used. This group shall be an untreated or sham treated control group or if a vehicle is used in administering the test substance, a vehicle control group.

(ii) If a vehicle is used in administering the test substance, the control group shall receive the vehicle in the highest volume used.

(iii) If a vehicle or other additive is used to facilitate dosing, it shall not interfere significantly with absorption of the test substance or produce toxic effects.

(3) *Dose levels and dose selection.* (i) At least three dose levels and a concurrent control shall be used.

(ii) The highest dose level should induce toxicity but not high levels of mortality in the parental (P) animals.

(iii) The lowest dose level should not produce any grossly observable evidence of toxicity.

(iv) Ideally the intermediate dose level(s) should produce minimal observable toxic effects. If more than one intermediate dose is used, dose levels should be spaced to produce a gradation of toxic effects.

(4) *Exposure conditions.* The animals should be dosed with the test substance, ideally, on a 7 days per week basis.

(i) Dosing, mating, delivery, and sacrifice schedule.

(A) Daily dosing of the parental (P) males and females shall begin when they are 5 to 8 weeks old. For both sexes, dosing shall be continued for at least 10 weeks before the mating period.

(B) Dosing of P males shall continue through the 3 week mating period. At the end of the mating period, P males may be sacrificed and examined, or may be retained for possible production of a second litter. If these animals are retained for a second litter, dosing shall be continued. Dosing of the $F_1$ males saved for mating shall continue from the time they are weaned through the period they are mated with the $F_1$ females (11 weeks). $F_1$ males may be sacrificed after the $F_1$ mating period.

(C) Daily dosing of the P females shall continue through the three week mating period, pregnancy, and to the weaning of the $F_1$ offspring. Dosing of the $F_1$ females saved for mating shall continue from the time they are weaned, through the period they are mated with the $F_1$ males (11 weeks from the time of weaning) pregnancy, and to the weaning of the $F_2$ offspring.

(ii) All animals are sacrificed as scheduled.

(A) All P males should be sacrificed at the end of the 3-week mating period, or may be retained for possible production of a second litter. If these animals are retained for a second litter, dosing shall be continued.

(B) $F_1$ males selected for mating should be sacrificed at the end of the three week period of the $F_1$ mating.

(C) $F_1$ males and females not selected for mating should be sacrified when weaned.

(D) The P females should be sacrificed upon weaning of their $F_1$ offspring.

(E) $F_1$ dams and their $F_2$ offspring are sacrificed when the offspring are weaned.

(5) *Administration of the test substance*—(i) *Oral studies.* (A) It is recommended that the test substance be administered in the diet or drinking water.

(B) If administered by gavage or capsule, the dosage administered to each animal prior to mating shall be based on the individual animal's body weight and adjusted weekly. During pregnancy the dosage shall be based on the body weight at day 0 and 6 of pregnancy.

(ii) If another route of administration is used, the tester should provide justification and reasoning for its selection.

(6) *Mating procedure*—(i) *Parental.* (A) For each mating, each female shall be placed with a single male from the same dose level until pregnancy occurs or 1 week has elapsed. If mating has not occurred after 1 week, the female shall be placed with a different male. Paired matings should be clearly identified.

(B) Those pairs that fail to mate should be evaluated to determine the cause of the apparent infertility. This may involve such procedures as additional opportunities to mate with proven fertile males or females, histological examination of the reproductive organs, and examination of the estrus or spermatogenic cycles.

(C) Each day, the females shall be examined for presence of sperm or vaginal plugs. Day 0 of pregnancy is defined as the day vaginal plugs or sperm are found.

(ii) *$F_1$ cross.* (A) For mating the $F_1$ offspring, one male and one female are randomly selected at weaning from each litter for cross mating with another pup of the same dose level but different litter, to produce the $F_2$ generation.

(B) $F_1$ males and females not selected for mating are sacrificed upon weaning.

(iii) *Special housing.* After evidence of copulation, pregnant animals shall be caged separately in delivery or maternity cages. Pregnant animals shall be provided with nesting materials when parturition is near.

(iv) *Standardization of litter sizes.* (A) On day 4 after birth, the size of each litter should be adjusted by eliminating extra pups by random selection to yield, as nearly as possible, 4 males and 4 females per litter.

(B) Whenever the number of male or female pups prevents having 4 of each sex per litter, partial adjustment (for example, 5 males and 3 females) is permitted. Adjustments are not appropriate for litters of less than 8 pups.

(C) Elimination of runts only is not appropriate.

(D) Adjustments of the $F_2$ litters is conducted in the same manner.

(7) *Observation of animals.* (i) A gross examination shall be made at least once each day. Pertinent behavioral changes, signs of difficult or prolonged parturition, and all signs of toxicity, including mortality, shall be recorded. These observations shall be reported for each individual animal. Food consumption for all animals shall be monitored weekly except during the mating period.

(ii) The duration of gestation shall be calculated from day 0 of pregnancy.

(iii) Each litter should be examined as soon as possible after delivery for the number of pups, stillbirths, live births, sex, and the presence of gross anomalies. Live pups should be counted and litters weighed at birth or soon thereafter, and on days 4, 7, 14, and 21 after parturition.

(iv) Physical or behavioral abnormalities observed in the dams of offspring shall be recorded.

(v) P males and females shall be weighed on the first day of dosing and weekly thereafter. $F_1$ litters shall be weighed at birth, or soon thereafter, and on days 4, 7, 14, and 21. In all cases, litter weights shall be calculated from the weights of the individual pups.

(8) *Gross necropsy.* (i) A complete gross examination shall be performed on all adult animals, including those which died during the experiment or were killed in moribund conditions.

(ii) Special attention shall be directed to the organs of the reproductive system.

(iii) The following organs and tissues, or representative samples thereof, shall be preserved in a suitable medium for possible future histopathological examination: Vagina; uterus; ovaries; testes; epididymides; seminal vesicles; prostate, pituitary gland; and, target organ(s) when previously identified of all P and $F_1$ animals selected for mating.

(9) *Histopathology.* Except if carried out in other studies of comparable duration and dose levels the following histopathology shall be performed:

(i) Full histopathology on the organs listed above for all high dose, and control $P_1$ and $F_1$ animals selected for mating.

(ii) Organs demonstrating pathology in these animals shall then be examined in animals from the other dose groups.

(iii) Microscopic examination shall be made of all tissues showing gross pathological changes.

(d) *Data and reporting*—(1) *Treatment of results.* Data shall be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals pregnant, the types of change and the percentage of animals displaying each type of change.

(2) *Evaluation of study results.* (i) An evaluation of test results, including the statistical analysis, based on the clinical findings, the gross necropsy findings, and the microscopic results shall be made and supplied. This should include an evaluation of the relationship, or lack thereof, between the animals' exposure to the test substance and the incidence and severity of all abnormalities.

(ii) In any study which demonstrates an absence of toxic effects, further investigation to establish absorption and bioavailability of the test substance should be considered.

(3) *Test report.* In addition to the reporting requirements as specified

under 40 CFR Part 792, Subpart J the following specific information shall be reported:

(i) Toxic response data by sex and dose, including fertility, gestation, viability and lactation indices, and length of gestation.

(ii) Species and strain.

(iii) Date of death during the study or whether animals survived to termination.

(iv) Toxic or other effects on reproduction, offspring, or postnatal growth.

(v) Date of observation of each abnormal sign and its subsequent course.

(vi) Body weight data for P, $F_1$, and $F_2$ animals.

(vii) Necropsy findings.

(viii) Detailed description of all histopathological findings.

(ix) Statistical treatment of results where appropriate.

(e) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Clermont, Y., Perry, B. "Quantitative Study of the Cell Population of the Seminiferous Tubules in Immature Rats," *American Journal of Anatomy.* 100:241-267 (1957).

(2) Goldenthal, E.I. *Guidelines for Reproduction Studies for Safety Evaluation of Drugs for Human Use.* Drug Review Branch, Division of Toxicological Evaluation, Bureau of Science, Food and Drug Administration, Washington, DC (1966).

(3) Hasegawa, T., Hayashi, M., Ebling, F.J.G., Henderson, I.W. *Fertility and Sterility.* (New York: American Elsevier Publishing Co., Inc., 1973).

(4) Oakberg, E.F. "Duration of Spermatogenesis in the Mouse and Timing of Stages of the Cycle of the Seminiferous Epithelium," *American Journal of Anatomy.* 9:507-516 (1956).

(5) Roosen-Runge, E.C. "The Process of Spermatogenesis in Mammals," *Biological Review.* 37:343-377 (1962).

[50 FR 39397, Sept. 27, 1985, as amended at 52 FR 19077, May 20, 1987]

### § 798.4900 Developmental toxicity study.

(a) *Purpose.* In the assessment and evaluation of the toxic characteristics of a chemical, determination of the potential developmental toxicity is important. The developmental toxicity study is designed to provide information on the potential hazard to the unborn which may arise from exposure of the mother during pregnancy.

(b) *Definitions.* (1) Developmental toxicity is the property of a chemical that causes in utero death, structural or functional abnormalities or growth retardation during the period of development.

(2) Dose is the amount of test substance administered. Dose is expressed as weight of test substance (g, mg) per unit weight of a test animal (e.g., mg/kg).

(3) No-observed-effect level is the maximum concentration in a test which produces no observed adverse effects. A no-observed-effect level is expressed in terms of weight of test substance given daily per unit weight of test animal (mg/kg)

(c) *Principle of the test method.* The test substance is administered in graduated doses for at least part of the pregnancy covering the major period of organogenesis, to several groups of pregnant experimental animals, one dose level being used per group. Shortly before the expected date of delivery, the pregnant females are sacrificed, the uteri removed, and the contents examined for embryonic or fetal deaths, and live fetuses.

(d) *Limit test.* If a test at an exposure of at least 1000 mg/kg body weight, using the procedures described for this study, produces no observable developmental toxicity, then a full study using three dose levels might not be necessary.

(e) *Test procedures*—(1) *Animal selection*—(i) *Species and strain.* Testing shall be performed in at least 2 mammalian species. Commonly used species include the rat, mouse, rabbit, and hamster. If other mammalian species are used, the tester shall provide justification/reasoning for their selection. Commonly used laboratory strains shall be employed. The strain shall not have low fecundity and shall preferably be characterized for its sensitivity to developmental toxins.

(ii) *Age.* Young adult animals (nulliparous females) shall be used.

(iii) *Sex.* Pregnant female animals shall be used at each dose level.

(iv) *Number of animals.* At least 20 pregnant rats, mice or hamsters or 12 pregnant rabbits are required at each dose level. The objective is to ensure that sufficient pups are produced to permit meaningful evaluation of the potential developmental toxicity of the test substance.

(2) *Control group.* A concurrent control group shall be used. This group shall be an untreated or sham treated control group, or, if a vehicle is used in administering the test substance, a vehicle control group. Except for treatment with the test substance, animals in the control group(s) shall be handled in an identical manner to test group animals.

(3) *Dose levels and dose selection.* (i) At least 3 dose levels with a control and, where appropriate, a vehicle control, shall be used.

(ii) The vehicle shall neither be developmentally toxic nor have effects on reproduction.

(iii) To select the appropriate dose levels, a pilot or trial study may be advisable. It is not always necessary to carry out a trial study in pregnant animals. Comparison of the results from a trial study in non-pregnant, and the main study in pregnant animals will demonstrate if the test substance is more toxic in pregnant animals. If a trial study is carried out in pregnant animals, the dose producing embryonic or fetal lethalities or maternal toxicity shall be determined.

(iv) Unless limited by the physical/chemical nature or biological properties of the substance, the highest dose level shall induce some overt maternal toxicity such as reduced body weight or body weight gain, but not more than 10 percent maternal deaths.

(v) The lowest dose level should not produce any grossly observable evidence of either maternal or developmental toxicity.

(vi) Ideally, the intermediate dose level(s) should produce minimal observable toxic effects. If more than one intermediate concentration is used, the concentration levels should be spaced to produce a gradation of toxic effects.

(4) *Observation period.* Day 0 in the test is the day on which a vaginal plug and/or sperm are observed. The dose period shall cover the period of major organogenesis. This may be taken as days 6 to 15 for rat and mouse, 6 to 14 for hamster, or 6 to 18 for rabbit.

(5) *Administration of test substance.* The test substance or vehicle is usually administered orally, by oral intubation unless the chemical or physical characteristics of the test substance or pattern of human exposure suggest a more appropriate route of administration. The test substance shall be administered approximately the same time each day.

(6) *Exposure conditions.* The female test animals are treated with the test substance daily throughout the appropriate treatment period. When given by gavage, the dose may be based on the weight of the females at the start of substance administration, or, alternatively, in view of the rapid weight gain which takes place during pregnancy, the animals may be weighed periodically and the dosage based on the most recent weight determination.

(7) *Observation of animals.* (i) A gross examination shall be made at least once each day.

(ii) Additional observations shall be made daily with appropriate actions taken to minimize loss of animals to the study (e.g., necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals).

(iii) Signs of toxicity shall be recorded as they are observed, including the time of onset, the degree and duration.

(iv) Cage-side observations shall include, but not be limited to: changes in skin and fur, eye and mucous membranes, as well as respiratory, autonomic and central nervous systems, somatomotor activity and behavioral pattern.

(v) Measurements should be made weekly of food consumption for all animals in the study.

(vi) Animals shall be weighed at least weekly.

(vii) Females showing signs of abortion or premature delivery shall be sacrificed and subjected to a thorough macroscopic examination.

(8) *Gross necropsy.* (i) At the time of sacrifice or death during the study, the dam shall be examined macroscopically for any structural abnormali-

ties or pathological changes which may have influenced the pregnancy.

(ii) Immediately after sacrifice or as soon as possible after death, the uterus shall be removed and the contents examined for embryonic or fetal deaths and the number of viable fetuses. The degree of resorption shall be described in order to help estimate the relative time of death of the conceptus. The weight of the gravid uterus should be recorded for dams that are sacrificed. Gravid uterine weights should not be obtained from dead animals if autolysis or decomposition has occurred.

(iii) The number of corpora lutea shall be determined for all species except mice.

(iv) The sex of the fetuses shall be determined and they shall be weighed individually, the weights recorded, and the mean fetal weight derived.

(v) Following removal, each fetus shall be examined externally.

(vi) For rats, mice and hamsters, one-third to one-half of each litter shall be prepared and examined for skeletal anomalies, and the remaining part of each litter shall be prepared and examined for soft tissue anomalies using appropriate methods.

(vii) For rabbits, each fetus shall be examined by careful dissection for visceral anomalies and then examined for skeletal anomalies.

(f) *Data and reporting*—(1) *Treatment of results.* Data shall be summarized in tablular form, showing for each test group: the number of animals at the start of the test, the number of pregnant animals, the number and percentages of live fetuses and the number of fetuses with any soft tissue or skeletal abnormalities.

(2) *Evaluation of results.* The findings of a developmental toxicity study shall be evaluated in terms of the observed effects and the exposure levels producing effects. It is necessary to consider the historical developmental toxicity data on the species/strain tested. A properly conducted developmental toxicity study should provide a satisfactory estimation of a no-effect level.

(3) *Test report.* In addition to the reporting requirements as specified under 40 CFR Part 792, Subpart J the following specific information shall be reported:

(i) Toxic response data by concentration.

(ii) Species and strain.

(iii) Date of death during the study or whether animals survived to termination.

(iv) Date of onset and duration of each abnormal sign and its subsequent course.

(v) Food, body weight and uterine weight data.

(vi) Pregnancy and litter data.

(vii) Fetal data (live/dead, sex, soft tissue and skeletal defects, resorptions).

(g) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Department of Health and Welfare. *The Testing of Chemicals for Carcinogenicity, mutagenicity and Teratogenicity.* Minister of Health and Welfare (Canada: Department of Health and Welfare, 1975).

(2) National Academy of Sciences. "Principles and Procedures for Evaluating the Toxicity of Household Substances." A report prepared by the Committee for the Revision of NAS Publication 1138, under the auspices of the Committee on Toxicology, National Research Council, National Academy of Sciences, Washington, DC (1977).

(3) World Health Organization. *Principles for the Testing of Drugs for Teratogenicity.* WHO Technical Report Series No. 364. (Geneva: World Health Organization, (1967).

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## Subpart F—Genetic Toxicity

### § 798.5100 Escherichia coli WP2 and WP2 uvrA reverse mutation assays.

(a) *Purpose.* The *E. coli* tryptophan (*trp*) reversion system is a microbial assay which measures trp–trp$^{+}$ reversion induced by chemicals which cause mutations in the genome of this organism.

(b) *Definition.* A reverse mutation assay in *E. coli* detects mutation in a gene of tryptophan requiring strain to produce a tryptophan independent strain of this organism.

(c) *Reference substances.* These may include, but need not be limited to, 4-nitroquinoline oxide, methyl methanesulfonate, or 2-aminoanthracene.

(d) *Test method*—(1) *Principle.* Bacteria are exposed to test chemical with and without metabolic activation and plated onto minimal medium. After a suitable period of incubation, revertant colonies are counted and compared to the number of spontaneous revertants in an untreated and/or vehicle control culture.

(2) *Description.* Several methods for performing the test have been described. Among those used are:

(i) The direct plate incorporation method.

(ii) The preincubation method.

(iii) The treat and plate method.

(iv) The modified fluctuation test.

(v) The desiccator method.

The procedure described here is for the direct plate incorporation method.

(3) *Strain selection*—(i) *Designation.* At the present time, three strains, WP2, WP2 *uvrA* and WP2 *uvrA*/pKM101 should be used. Other strains may be utilized when appropriate.

(ii) *Preparation and storage.* Recognized methods of stock culture preparation and storage should be used. The requirement of tryptophan for growth should be demonstrated for each strain. Other phenotypic characteristics should be checked using such methods as sensitivity to mitomycin C and resistance to ampicillin. Spontaneous reversion frequency should be in the range expected either as reported in the literature or as established in the laboratory by historical control values.

(iii) *Bacterial growth.* Fresh cultures of bacteria should be grown up to the late exponential or early stationary phase of growth (approximately $10^{8}$-$10^{9}$ cells per ml).

(4) *Metabolic activation.* Bacteria should be exposed to the test substance both in the presence and absence of an appropriate metabolic activation system. The most commonly used system is a cofactor supplemented postmitochondrial fraction prepared from the livers of rodents treated with enzyme inducing agents. The use of other species, tissues or techniques may also be appropriate.

(5) *Control groups*—(i) *Concurrent controls.* Concurrent positive and negative (untreated and/or vehicle) controls should be included in each experiment.

(ii) *Direct acting positive controls.* Examples of positive controls for assays performed without metabolic activation include methyl methanesulfonate and 4-nitroquinoline oxide.

(iii) *Positive controls to ensure the efficacy of the activation system.* The positive control reference substance for tests including a metabolic activation system should be selected on the basis of the type of activation system used in the test. 2-Aminoanthracene is an example of a positive control compound in tests using postmitochondrial fractions from the livers of rodents treated with enzyme inducing agents such as Aroclor-1254.

(iv) *Other positive controls.* Other positive control reference substances may be used.

(6) *Test chemicals*—(i) *Vehicle.* Test chemicals and positive control reference substances should be dissolved in an appropriate vehicle and then further diluted in vehicle for use in the assay.

(ii) *Exposure concentrations.* (A) The test should initially be performed over a broad range of concentrations. Among the criteria to be taken into consideration for determinating the upper limits of test chemical concentration are cytotoxicity and solubility. Cytotoxicity of the test chemical may be altered in the presence of metabolic activation systems. Toxicity may be evidenced by a reduction in the number of spontaneous revertants, a clearing of the background lawn or by the degree of survival of treated cultures. Relatively insoluble chemicals should be tested up to the limits of solubility. For freely soluble nontoxic chemicals, the upper test chemical concentration should be determined on a case by case basis.

(B) Generally, a maximum of 5 mg/plate for pure substances is considered acceptable. At least 5 different amounts of test substance should be tested with adequate intervals between the test points.

(C) When appropriate, a positive response should be confirmed by testing over a narrow range of concentrations.

(e) *Test performance*—(1) *Direct plate incorporation method.* (i) For this test without metabolic activation, test chemical and 0.1 ml of a fresh bacterial culture should be added to 2.0 ml of overlay agar.

(ii) For tests with metabolic activation, 0.5 ml of activation mixture containing an adequate amount of postmitochondrial fraction should be added to the overlay agar after the addition of test chemical and bacteria. Contents of each tube should be mixed and poured over the surface of a selective agar plate. Overlay agar should be allowed to solidify before incubation. At the end of the incubation period, revertant colonies per plate should be counted.

(2) *Other methods.* Other methods may also be appropriate.

(3) *Media.* An appropriate selective medium with an adequate overlay agar should be used.

(4) *Incubation conditions.* All plates in a given experiment should be incubated for the same time period. This incubation period should be for 48-72 hours at 37 °C.

(5) *Number of cultures.* All plating should be done at least in triplicate. If scientifically justified, the use of duplicates may be acceptable. All results should be confirmed in an independent experiment.

(f) *Data and report*—(1) *Treatment of results.* Data should be presented as number of revertant colonies per plate for each replicate and dose. The numbers of revertant colonies on both negative (untreated and/or vehicle) and positive control plates should also be presented. Individual plate counts, the mean number of revertant colonies per plate and standard deviation should be presented for test chemical and positive and negative (untreated and/or vehicle) controls.

(2) *Statistical evaluation.* Data should be evaluated by appropriate statistical methods.

(3) *Interpretation of results.* (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of revertants. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test substance concentrations.

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of revertants or a statistically significant and reproducible positive response at any one of the test points is considered non-mutagenic in the system.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(4) *Test evaluation.* (i) Positive results from the *E. coli* reverse mutation assay indicate that, under the test conditions, the test substance induces mutations in the genome of this organism.

(ii) Negative results indicate that under the test conditions the test substance is not mutagenic in *E. coli.*

(5) *Test report.* In addition to the reporting recommendations as specified under 40 CFR Part 792, Subpart J the following specific information should be reported:

(i) Bacterial strain used.

(ii) Details of both the protocol used to prepare the metabolic activation system and of its use in the assay.

(iii) Dose levels and rationale for selection of dose.

(iv) Positive and negative controls.

(v) Individual plate counts, mean number of revertant colonies per plate, standard deviation.

(vi) Dose-response relationship, if applicable.

(g) *References.* For additional background information on this test guideline, the following references should be consulted:

(1) Ames, B.N. McCann, J., Yamasaki, E. "Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test," *Mutation Research* 31:347-364 (1975).

(2) Brusick, D.J., Simmon, V.F., Rosenkranz, H.S., Ray, V.A., Stafford, R.S. An evaluation of the *Escherichia coli* WP2 and WP2 *uvrA* reverse mutation assay," *Mutation Research* 76:169-190 (1980).

(3) Green, M.H.L., Muriel, W.J. "Mutagen testing using trp⁺ in *Escherichia coli*," *Mutation Research* 38:3–32 (1976).

(4) Vogel, H.J., Bonner, D.M. "Acetylornithinase of *E. coli*: partial purification and some properties," *Journal of Biological Chemistry* 218:97–106 (1956).

### § 798.5140 Gene mutation in aspergillus nidulans.

(a) *Purpose. Aspergillus nidulans* is a eukaryotic fungus which has been developed to detect and study a variety of genetic phenomena including chemically induced mutagenesis. *A. nidulans* can be used to detect both forward and reverse gene mutation. These mutations are detected by changes in colonial morphology or nutritional requirements in treated populations. The methionine and 2-thioxanthine forward mutation systems can be used to detect mutations in *A. nidulans.*

(b) *Definition.* A forward mutation is a gene mutation from the wild (parent) type to the mutant condition.

(c) *Reference substances.* These may include, but need not be limited to, ethyl methanesulfonate, cyclophosphamide or aflatoxin $B_1$.

(d) *Test method*—(1) *Principle.* Conidia are exposed to test chemical both with and without metabolic activation and plated on selective medium to determine changes in colonial morphology or nutritional requirements. At the end of a suitable incubation period, mutant colonies are counted and compared to the number of spontaneous mutants in an untreated control culture. Simultaneous determination of survival permits calculation of mutation frequency.

(2) *Description.* Tests for mutation in *A. nidulans* are performed in liquid suspension. Treated conidia are plated on selective medium to determine changes in nutritional requirements or colonial morphology.

(3) *Strain selection*—(i) *Designation.* For the methionine and 2-thioxanthine systems the hapliod Glascow *biAl; meth G1* strain is the most commonly used strain although other strains may be appropriate. Any translocation-free strain which produces green colonies on thioxanthine free medium and yellow colonies on medium containing thioxanthine may be used in the thioxanthine system.

(ii) *Preparation and storage.* Stock culture preparation and storage, growth requirements, method of strain identification and demonstration of appropriate phenotypic requirements should be performed using good microbiological techniques and should be documented.

(iii) *Media.* Any medium which supports growth and a characteristic colonial morphology may be used in the assay.

(4) *Preparation of conidia.* Prior to chemical treatment, conidia from 4–5 single colonies of the appropriate strain are grown at 37 °C on complete medium. At the end of the incubation period, conidia are collected, conidial chains broken up, mycelial debris removed and conidia concentrated prior to removal of the germination inhibitory substance. Germination inhibitory substance should be removed by Tween 80 or diethyl ether.

(5) *Metabolic activation.* Conidia should be exposed to test substance both in the presence and absence of an appropriate metabolic activation system.

(6) *Control groups.* Concurrent positive and negative untreated and/or vehicle) controls both with and without metabolic activation should be included in each experiment.

(7) *Test chemicals*—(i) *Vehicle.* Test chemicals and positive control reference substances should be dissolved in an appropriate vehicle and then further diluted in vehicle for use in the assay.

(ii) *Exposure concentrations.* (A) The test should initially be performed over a broad range of concentrations selected on the basis of a preliminary assay. Effective treatment times should also be selected in the preliminary assay.

(B) Each test should include five treatment points, two at fixed concentrations for different time periods, and three at varying concentrations for fixed periods of time.

(C) Among the criteria to be taken into consideration for determining the upper limits of test chemical concentration are cytotoxicity and solubility. Cytotoxicity of the test chemical may be altered in the presence of a metabolic activation system. Relatively insoluble chemicals should be tested up to the limits of solubility. For freely soluble nontoxic chemicals, the upper test chemical concentration should be determined on a case by case basis.

(D) When appropriate, a positive response should be confirmed by using a narrow range of test concentrations.

(e) *Test Performance*—(1) *Treatment.* Germinating or quiescent conidia in liquid suspension should be exposed to the test chemical at 37 °C under conditions of yellow light and controlled pH and oxygen tension. At the end of the exposure period, treatment should be terminated by repeated centrifugation and washing of the conidia or by dilution. Chemical neutralization of the test agent may also be used but is not recommended.

(2) *Media*—(i) *Methionine system.* For the methionine system, conidia should be plated on methionine deficient medium for mutant selection and on medium supplemented with methionine to determine survival.

(ii) *Thioxanthine system.* (A) For the 2-thioxanthine system, treated conidia should be plated on nitrogen-free glucose and salts minimal medium containing 2-thioxanthine.

(B) After incubation, green colonies should be counted and isolated by restreaking. The isolated colonies should be classified on the basis of genetic criteria. Yellow, wild-type colonies will grow on the same plate. This permits concurrent determination of survival and an estimation of mutation frequency.

(3) *Determination of mutation frequency and viability.* In both systems, mutation frequency and viability should be determined immediately before and immediately after chemical treatment.

(4) *Incubation conditions.* All incubations should be at [illegible] C. Incubation time will vary depending upon system and endpoint (mutation or viability) being determined.

(5) *Number of cultures.* (i) At least 10 independent plates per concentration with no more than 20 colonies per plate should be used in the methionine system.

(ii) Fifteen to 20 plates per concentration are preferred for the 2-thioxanthine system.

(f) *Data and report*—(1) *Treatment of results.* Individual plate counts for test substance and controls should be presented for both mutation induction and survival. The mean number of colonies per plate and standard deviation should also be presented. Data should be presented in tabular form indicating, as applicable, numbers of colonies counted, and numbers and classification of mutants identified. Sufficient detail should be provided for verification of survival and mutation frequencies.

(2) *Statistical evaluation.* Data should be evaluated by appropriate statistical methods.

(3) *Interpretation of results.* (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of mutant colonies. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test points.

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of mutant colonies or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(4) *Test evaluation.* (i) Positive results from the methionine and 2-thioxanthine systems in *A. nidulans* indicate that, under the test conditions, the test substance causes gene (point) mutations in the DNA of this organism caused by base pair changes and small deletions in the genome.

(ii) Negative results indicate that under the test conditions the test chemical is not mutagenic in *A. nidulans.*

(5) *Test report.* In addition to the reporting recommendations as specified under 40 CFR Part 792, Subpart J the

following specific information should be reported:

(i) Strain of organism used in the assay.

(ii) Test chemical vehicle, doses used and rationale for dose selection, toxicity data.

(iii) Method used for preparation of conidia.

(iv) Treatment conditions, including length of exposure and method used to stop treatment.

(v) Details of both the protocol used to prepare the metabolic activation system and of its use in the assay.

(vi) Incubation times and temperature.

(vii) Positive and negative controls.

(viii) Dose-response relationship, if applicable.

(g) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Ames, B.N., McCann, J., Yamasaki, E. "Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test," *Mutation Research* 31:347–364 (1975).

(2) Kafer, E., Scott, B.R., Dorn, G.L., Stafford, R.S. "*Aspergillus nidulans:* systems and results of tests for chemical induction of mitotic segregation and mutation. I. Diploid and duplication assay systems: a report of the U.S. EPA's Gene-Tox Program," *Mutation Research* 98:1–48 (1982).

(3) Munson, R.J., Goodhead, D.T. "Relation between induced mutation frequency and cell survival: a theoretical approach and an examination of experimental data for eukaryotes," *Mutation Research* 42:145–159 (1977).

(4) Scott, B.R., Dorn, G.L., Kafer, E., Stafford, R.S. "*Aspergillus nidulans:* systems and results of tests for mitotic segregation and mutation. II. Haploid assay systems and overall response of all systems: a report of the U.S. EPA's Gene-Tox Program," *Mutation Research* 98:49–94 (1982).

### § 798.5200 Mouse visible specific locus test.

(a) *Purpose.* The mouse visible specific locus test (MSLT) may be used to detect and quantitate mutations in the germ line of a mammalian species.

(b) *Definitions.* (1) A visible specific locus mutation is a genetic change that alters factors responsible for coat color and other visible characteristics of certain mouse strains.

(2) The germ line is the cells in the gonads of higher eukaryotes which are the carriers of the genetic information for the species.

(c) *Reference substances.* Not applicable.

(d) *Test method*—(1) *Principle.* (i) The principle of the MSLT is to cross individuals who differ with respect to the genes present at certain specific loci, so that a genetic alteration involving the standard gene at any one of these loci will produce an offspring detectably different from the standard heterozygote. The genetic change may be detectable by various means, depending on the loci chosen to be marked.

(ii) Three variations of the method currently exist for detecting newly arising point mutations in mouse germ cells:

(A) The visible specific locus test using either 5 or 7 loci.

(B) The biochemical specific locus test using up to 20 enzymes.

(C) The test for mutations at histocompatibility loci.

(iii) Of the three tests, the visible specific locus test has been most widely used in assessing genetic hazard due to environmental agents. It is the method described in this guideline.

(2) *Description.* For technical reasons, males rather than females are generally treated with the test agent. Treated males are then mated to females which are genetically homozygous for certain specific visible marker loci. Offspring are examined in the next generation for evidence that a new mutation has arisen.

(3) *Animal selection*—(i) *Species and strain.* Mice shall be used as the test species. Male mice shall be either $(C_3H \times 101)F_1$ or $(101 \times C_3H)F_1$ hybrids. Females shall be T stock virgins.

(ii) *Age.* Healthy sexually mature animals shall be used.

(iii) *Number.* A decision on the minimum number of treated animals should take into account the spontaneous variation of the biological characterization being evaluated. Other considerations should include:

(A) The use of either historical or concurrent controls.

(B) The power of the test.

(C) The minimal rate of induction required.

(D) The use of positive controls.

(E) The level of significance desired.

(iv) *Assignment to groups.* Animals shall be randomized and assigned to treatment and control groups.

(4) *Control groups*—(i) *Concurrent controls.* The use of positive or spontaneous controls is left to the discretion of the investigator. However, any laboratory which has had no prior experience with the test shall, at its first attempt, produce a negative control sample of 20,000 and a positive control, using 100 mg/kg 1-ethyl-nitrosourea, in a sample of 5,000 offspring.

(ii) *Historical controls.* Long term, accumulated spontaneous control data of 43/801,406 are available for comparative purposes.

(5) *Test chemicals*— (i) *Vehicle.* When possible, test chemicals should be dissolved or suspended in distilled water or isotonic saline buffered appropriately, if needed, for stability. Water-insoluble chemicals shall be dissolved or suspended in appropriate vehicles. The vehicle used shall neither interfere with the test compound nor produce major toxic effects. Fresh preparations of the test chemical should be employed.

(ii) *Dose levels.* Usually, only one dose level need be tested. This should be the highest dose tolerated without toxic effects, provided that any temporary sterility induced due to elimination of spermatagonia is of only moderate duration, as determined by a return of males to fertility within 80 days after treatment. For evaluation of dose-response, it is recommended that at least two dose levels be tested.

(iii) *Route of administration.* Acceptable routes of administration include gavage, inhalation, admixture with food or water, and IP or IV injections.

(e) *Test performance*—(1) *Treatment and mating.* Hybrid $F_1$ ($C_3H \times 101$ or $101 \times C_3H$) male mice shall be treated with the test substance and immediately mated to virgin T stock females. Each treated male shall be mated to a fresh group of 2 to 4 virgin females each week for 7 weeks, after which he shall be returned to the first group of females and rotated through the seven sets of females repeatedly. This mating schedule generally permits sampling of all postspermatagonial stages of germ cell development during the first 7 weeks and rapid accumulation of data for exposed spermatagonial stem cells thereafter. Repeated mating cycles should be conducted until the entire spermatogonial cycle has been evaluated and enough offspring have been obtained to meet the power criterion of the assay.

(2) *Examination of offspring.* (i) Offspring may be examined at (or soon after) birth but must be examined at about 3 weeks of age at which time the numbers of mutant and nonmutant offspring in each litter shall be recorded.

(ii) Nonmutant progeny should be discarded. Mutant progeny shall be subjected to genetic tests for verification.

(f) *Data and report*—(1) *Treatment of results.* Data shall be presented in tabular form and shall permit independent analysis of cell stage specific effects and dose dependent phenomena. The data shall be recorded and analyzed in such a way that clusters of identical mutations are clearly identified. The individual mutants detected shall be thoroughly described. In addition, concurrent positive and negative control data, if they are available, shall be tabulated so that it is possible to differentiate between concurrent (when available) and long-term accumulated mutation frequencies.

(2) *Statistical evaluation.* Data shall be evaluated by appropriate statistical methods.

(3) *Interpretation of results.* (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of specific locus mutations. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test points.

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of specific locus mutations or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(4) *Test evaluation.* (i) Positive results in the MSLT indicate that under the test conditions the test substance induces heritable gene mutations in the test species.

(ii) Negative results indicate that under the test conditions the test substance does not induce heritable gene mutations in the test species.

(5) *Test report.* In addition to the reporting recommendations as specified under 40 CFR Part 792, Subpart J the following specific information shall be reported:

(i) Strain, age and weight of animals used, number of animals of each sex in experimental and control groups.

(ii) Test chemical vehicle, doses used and rationale for dose selection, toxicity data.

(iii) Route and duration of exposure.

(iv) Mating schedule.

(v) Time of examination for mutant progeny.

(vi) Criteria for scoring mutants.

(vii) Use of concurrent or negative controls.

(viii) Dose response relationship, if applicable.

(g) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Russell, L.B., Shelby, P.B., von Halle, E., Sheridan, W., Valcovic, L. The mouse specific locus test with agents other than radiations: interpretation of data and recommendations for future work: A report of the U.S. EPA's Gene-Tox Program," *Mutation Research*, 86:329-354 (1981).

(2) [Reserved]

[50 FR 39397, Sept. 27, 1985, as amended at 52 FR 19078, May 20, 1987]

## § 798.5250 Gene mutation in neurospora crassa.

(a) *Purpose. Neurospora crassa* is a eukaryotic fungus which has been developed to detect and study a variety of genetic phenomena including chemically induced mutagenesis. *N. crassa* can be used to detect both forward and reverse gene mutation. These mutations are detected by biochemical or morphological changes in the treated population. The most commonly used mutation assay in *N. crassa* measures forward mutation in the *ad-3* region of the genome.

(b) *Definition.* A forward mutation is a gene mutation from the wild (parent) type to the mutant condition.

(c) *Reference substances.* These may include, but need not be limited to, ethyl- or methyl methanesulfonate.

(d) *Test method*—(1) *Principle.* The detection of forward mutations at the *ad-3* locus in either homokaryons or heterokaryons may be used. However, use of two component heterokaryons is recommended because of the greater range of mutations which can be recovered. In either case, the test relies on the identification of purple (mutant) colonies among a large number of white (wild-type) colonies. A representative sample of purple colonies can be recovered and thoroughly analyzed genetically.

(2) *Description.* Forward mutations at the *ad-3* locus can be detected using noncolonial strains of *N. crassa* grown on media containing sorbose as well as glucose. Under these conditions, colonies are formed and reproducible colonial morphology results. Adenine-requiring mutants which accumulate a reddish-purple pigment can be readily identified and counted.

(3) *Strain selection*—(i) *Designation.* At the present time, heterokaryon 12 is recommended for use in this assay. The use of other strains may also be appropriate.

(ii) *Preparation and storage.* Stock culture preparation and storage, growth requirements, method of strain identification and demonstration of appropriate phenotypic requirements should be performed using good microbiological techniques and should be documented.

(iii) *Media.* Frie's No. 3 minimal medium or Westgaard's Synthetic medium with 1.5 percent agar or any medium known to support growth and characteristic colonial morphology may be used in the assay.

(4) *Preparation of conidia.* Stock cultures should be grown on minimal medium to select for single colonies with noncolonial morphology. Single colony isolates then should be inoculated into agar flasks and incubated at 35 C for 48 hrs to select colonies with spreading growth patterns in which mycelia cover the entire flask. Flasks should be incubated at 23-25 C and those with bright orange conidia selected for preparation of conidial suspensions. Suspensions should be diluted for use in distilled water.

(5) *Metabolic activation.* Conidia should be exposed to test substance both in the presence and absence of an appropriate metabolic activation system.

(6) *Control groups.* Concurrent positive and negative (untreated and/or vehicle) controls both with and without metabolic activation should be included in each experiment.

(7) *Test chemicals*—(i) *Vehicle.* Test chemicals and positive control reference substances should be dissolved in an appropriate vehicle and then further diluted in vehicle for use in the assay.

(ii) *Exposure concentrations.* (A) The test should initially be performed over a broad range of concentrations selected on the basis of a preliminary assay. Effective treatment times should also be selected in the preliminary assay.

(B) Among the criteria to be taken into consideration for determining the upper limits of test chemical concentration are cytotoxicity and solubility. Cytotoxicity of the test chemical may be altered in the presence of metabolic activation systems. For toxic chemicals, the highest concentration tested should not reduce survival below 10 percent of that seen in the control cultures. Relatively insoluble chemicals should be tested up to the limits of solubility. For freely soluble nontoxic chemicals, the upper test chemical concentration should be determined on a case by case basis.

(C) Each test should include five treatment points; two at fixed concentrations for different time periods, and three at varying concentrations for fixed periods of time.

(D) When appropriate, a positive response should be confirmed by testing over a narrow range of concentrations.

(e) *Test performance*—(1) *Treatment.* (i) Growing or nongrowing conidia should be exposed to the test chemical with and without metabolic activation. At the end of the exposure period, treatment should be terminated by chemical quenching. The quenching solution may contain 0.1 percent sodium thiosulfate.

(ii) Conidia should then be plated on the appropriate media to determine mutation induction and viability. At the end of the incubation period, colonies should be scored for viability and mutation induction.

(iii) Mutants should be classified according to color and morphology.

(iv) Both mutation frequency and viability should be determined both immediately before and immediately after chemical treatment.

(2) *Incubation conditions.* All plates in a given test should be incubated for the same time period. This incubation period may be from 2 to 7 days at 30 °C.

(3) *Number of cultures.* Generally, 15 to 20 individual plates per concentration should be used.

(f) *Data and report*—(1) *Treatment of results.* Individual plate counts for test substance and controls should be presented for both mutation induction and survival. The mean number of colonies per plate and standard deviation should be presented. Data should be presented in tabular form indicating, as applicable, numbers of colonies counted, numbers of mutants identified and classification of mutants (e.g., color segregants). Sufficient detail should be provided for verification of survival and mutation frequencies.

(2) *Statistical evaluation.* Data should be evaluated by appropriate statistical techniques.

(3) *Interpretation of results.* (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of mutant colonies. Another criterion may be based upon detection of a reproducible and statistically significant positive re-

sponse for at least one of the test points.

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of mutant colonies or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(4) *Test evaluation.* (i) Positive results from the *ad-3* system in *N. crassa* indicate that, under the test conditions, the test substance causes mutations in the DNA of this organism.

(ii) Negative results indicate that under the test conditions the test substance is not mutagenic in *N. crassa.*

(5) *Test report.* In addition to the reporting recommendations as specified under 40 CFR Part 792, Subpart J the following specific information should be reported:

(i) Strain of organism used in the assay.

(ii) Test chemical vehicle, doses used and rationale for dose selection.

(iii) Method used for preparation of conida.

(iv) Treatment conditions, including length of exposure and method used to stop treatment.

(v) Incubation times and temperature.

(vi) Details of both the protocol used to prepare the metabolic activation system and of its use in the assay.

(vii) Dose-response relationship, if applicable.

(g) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Brockman, H.E., de Serres, F.J. "Induction of *ad-3* mutants of *Neurospora crassa* by 2-aminopurine," *Genetics* 48: 597-604 (1963).

(2) de Serres, F.J., Malling, H.V. "Measurement of recessive lethal damage over the entire genome and at two specific loci in the *ad-3* region of a two-component heterokaryon of *Neurospora crassa,*" *Chemical mutagens: principles and methods for their detection.* Vol. 2, Ed. Hollaender, A. (New York and London: Plenum Press, 1971) pp. 311-342.

(3) Matzinger, P.K. Ong, T-M. "In vitro activation of aflatoxin $B_1$ to metabolites mutagenic in *Neurospora crassa.*" *Mutation Research,* 37:27-32 (1976).

### § 798.5265 The salmonella typhimurium reverse mutation assay.

(a) *Purpose.* The *Salmonella typhimurium* histidine (his) reversion system is a microbial assay which measures $his^-$ → $his^+$ reversion induced by chemicals which cause base changes or frameshift mutations in the genome of this organism.

(b) *Definitions.* (1) A reverse mutation assay in *Salmonella typhimurium* detects mutation in a gene of a histidine requiring strain to produce a histidine independent strain of this organism.

(2) Base pair mutagens are agents which cause a base change in the DNA. In a reversion assay, this change may occur at the site of the original mutation or at a second site in the chromosome.

(3) Frameshift mutagens are agents which cause the addition or deletion of single or multiple base pairs in the DNA molecule.

(c) *Reference substances.* These may include, but need not be limited to, sodium azide, 2-nitrofluorene, 9-aminoacridine, 2-aminoanthracene, congo red, benzopurpurin 4B, trypan blue or direct blue 1.

(d) *Test method*—(1) *Principle.* Bacteria are exposed to test chemical with and without a metabolic activation system and plated onto minimal medium. After a suitable period of incubation, revertant colonies are counted and compared to the number of spontaneous revertants in an untreated and/or vehicle control culture.

(2) *Description.* Several methods for performing the test have been described. Among those used are:

(i) The direct plate incorporation method.

(ii) The preincubation method.

(iii) The azo-reduction method.

The procedures described here are for the direct plate incorporation method and the azo-reduction method.

(3) *Strain selection*—(i) *Designation.* At the present time four strains, TA 1535, TA 1537, TA 98 and TA 100 should be used. The use of other strains in addition to these four is left to the discretion of the investigator.

(ii) *Preparation and storage.* Recognized methods of stock culture preparation and storage should be used. The requirement of histidine for growth should be demonstrated for each strain. Other phenotypic characteristics should be checked using such methods as crystal violet sensitivity and resistance to ampicillin. Spontaneous reversion frequency should be in the range expected either as reported in the literature or as established in the laboratory by historical control values.

(iii) *Bacterial growth.* Fresh cultures of bacteria should be grown up to the late exponential or early stationary phase of growth (approximately $10^8$-$10^9$ cells per ml).

(4) *Metabolic activation.* Bacteria should be exposed to the test substance both in the presence and absence of an appropriate metabolic activation system. For the direct plate incorporation method, the most commonly used system is a cofactor supplemented postmitochondrial fraction prepared from the livers of rodents treated with enzyme inducing agents such as Aroclor 1254. For the azo-reduction method, a cofactor supplemented postmitochondrial fraction prepared from the livers of untreated hamsters is preferred. For this method, the cofactor supplement should contain flavin mononucleotide, exogenous glucose 6-phosphate dehydrogenase, NADH and excess of glucose-6-phosphate.

(5) *Control groups*—(i) *Concurrent controls.* Concurrent positive and negative (untreated and/or vehicle) controls shall be included in each experiment. Positive controls shall ensure both strain responsiveness and efficacy of the metabolic activation system.

(ii) *Strain specific positive controls.* Strain specific positive controls shall be included in the assay. Examples of strain specific positive controls are as follows:

(A) Strain TA 1535, TA 100, sodium azide.

(B) TA 98, 2-nitrofluorene.

(C) TA 1537, 9-aminoacridine.

(iii) *Positive controls to ensure the efficacy of the activation system.* The positive control reference substance for tests including a metabolic activation system should be selected on the basis of the type of activation system used in the test. 2-Aminoanthracene is an example of a positive control compound in plate-incorporation tests using postmitochondrial fractions from the livers of rodents treated with enzyme inducing agents such as Aroclor-1254. Congo red is an example of a positive control compound in the azo-reduction method. Other positive control reference substances may be used.

(iv) *Class-specific positive controls.* The azo-reduction method should include positive controls from the same class of compounds as the test agent wherever possible.

(6) *Test chemicals*—(i) *Vehicle.* Test chemicals and positive control reference substances should be dissolved or suspended in an appropriate vehicle and then further diluted in vehicle for use in the assay.

(ii) *Exposure concentrations.* (A) The test should initially be performed over a broad range of concentrations. Among the criteria to be taken into consideration for determining the upper limits of test chemical concentration are cytotoxicity and solubility. Cytotoxicity of the test chemical may be altered in the presence of metabolic activation systems. Toxicity may be evidenced by a reduction in the number of spontaneous revertants, a clearing of the background lawn or by the degree of survival of treated cultures. Relatively insoluble compounds should be tested up to the limits of solubility. For freely soluble nontoxic chemicals, the upper test chemical concentration should be determined on a case by case basis.

(B) Generally, a maximum of 5 mg/plate for pure substances is considered acceptable. At least 5 different amounts of test substance shall be tested with adequate intervals between test points.

(C) When appropriate, a single positive response shall be confirmed by testing over a narrow range of concentrations.

(e) *Test performance*—(*1*) *Direct plate incorporation method*. For this test without metabolic activation, test chemical and 0.1 ml of a fresh bacterial culture should be added to 2.0 ml of overlay agar. For tests with metabolic activation, 0.5 ml of activation mixture containing an adequate amount of postmitochondrial fraction should be added to the agar overlay after the addition of test chemical and bacteria. Contents of each tube shall be mixed and poured over the surface of a selective agar plate. Overlay agar shall be allowed to solidify before incubation. At the end of the incubation period, revertant colonies per plate shall be counted.

(2) *Azo-reduction method*. (i) For this test with metabolic activation, 0.5 ml of S-9 mix containing 150 ul of S-9 and 0.1 ml of bacterial culture should be added to a test tube kept on ice. One-tenth milliliter of chemical should be added, and the tubes should be incubated with shaking at 30° C for 30 min. At the end of the incubation period, 2.0 ml of agar should be added to each tube, the contents mixed and poured over the surface of a selective agar plate. Overlay agar shall be allowed to solidify before incubation. At the end of the incubation period, revertant colonies per plate shall be counted.

(ii) For tests without metabolic activation, 0.5 ml of buffer should be used in place of the 0.5 ml of S-9 mix. All other procedures shall be the same as those used for the test with metabolic activation.

(3) *Other methods*. Other methods may also be appropriate.

(4) *Media*. An appropriate selective medium with an adequate overlay agar shall be used.

(5) *Incubation conditions*. All plates within a given experiment shall be incubated for the same time period. This incubation period shall be for 48-72 hours at 37 °C.

(6) *Number of cultures*. All plating should be done at least in triplicate.

(f) *Data and report*—(1) *Treatment of results*. Data shall be presented as number of revertant colonies per plate for each replicate and dose. The numbers of revertant colonies on both negative (untreated and/or vehicle) and positive control plates shall also be presented. Individual plate counts, the mean number of revertant colonies per plate and standard deviation shall be presented for test chemical and positive and negative (untreated and/or vehicle) controls.

(2) *Statistical evaluation*. Data should be evaluated by appropriate statistical methods.

(3) *Interpretation of results*. (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of revertants. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test substance concentrations.

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of revertants or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(4) *Test evaluation*. (i) Positive results from the *S. typhimurium* reverse mutation assay indicate that, under the test conditions, the test substance induces point mutations by base changes or frameshifts in the genome of this organism.

(ii) Negative results indicate that under the test conditions the test substance is not mutagenic in *S. typhimurium*.

(5) *Test report*. In addition to the reporting recommendations as specified under 40 CFR Part 792, Subpart J, the following specific information shall be reported:

(i) Bacterial strain used.

(ii) Metabolic activation system used (source, amount and cofactor); details of preparations of S-9 mix.

(iii) Dose levels and rationale for selection of dose.

(iv) Positive and negative controls.

(v) Individual plate counts, mean number of revertant colonies per plate, standard deviation.

(vi) Dose-response relationship, if applicable.

(g) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Ames, B.N., McCann, J., Yamasaki, E. "Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test," *Mutation Research* 31:347-364 (1975).

(2) de Serres, F.J., Shelby, M.D. "The *Salmonella* mutagenicity assay: recommendations," *Science* 203:563-565 (1979).

(3) Prival, M.J., Mitchell, V.D. "Analysis of a method for testing azo dyes for mutagenic activity in *Salmonella typhimurium* in the presence of flavin mononucleotide and hamster liver S-9," *Mutation Research* 97:103-116 (1982).

(4) Vogel, H.J., Bonner, D.M. "Acetylornithinase of *E. coli*: partial purification and some properties," *Journal of Biological Chemistry.* 218:97-106 (1956).

[50 FR 39397, Sept. 27, 1985, as amended at 52 FR 19078, May 20, 1987]

### § 798.5275 Sex-linked recessive lethal test in drosophila melanogaster.

(a) *Purpose.* The sex-linked recessive lethal (SLRL) test using *Drosophila melanogaster* detects the occurrence of mutations, both point mutations and small deletions, in the germ line of the insect. This test is a forward mutation assay capable of screening for mutations at about 800 loci on the X-chromosome. This represents about 80 percent of all X-chromosome loci. The X-chromosome represents approximately one-fifth of the entire haploid genome.

(b) *Definitions.* (1) Lethal mutation is a change in the genome which, when expressed, causes death to the carrier.

(2) Recessive mutation is a change in the genome which is expressed in the homozygous or hemizygous condition.

(3) Sex-Linked genes are present on the sex (X or Y) chromosomes. Sex-linked genes in the context of this guideline refer only to those located on the X-chromosome.

(c) *Reference substances.* These may include, but need not be limited to, ethyl methanesulfonate or N-nitrosodimethylamine.

(d) *Test method*—(1) *Principle.* Mutations in the X-chromosome of *D. melanogaster* are phenotypically expressed in males carrying the mutant gene. When the mutation is lethal in the hemizygous condition, its presence is inferred from the absence of one class of male offspring out of the two that are normally produced by a heterozygous female. The SLRL test takes advantage of these facts by means of specially marked and arranged chromosomes.

(2) *Description.* Wild-type males are treated and mated to appropriate females. Female offspring are mated individually to their brothers, and in the next generation the progeny from each separate dose are scored for phenotypically wild-type males. Absence of these males indicates that a sex-linked recessive lethal mutation has occurred in a germ cell of the $P_1$ male.

(3) *Drosophila stocks.* Males of a well-defined wild type stock and females of the Muller-5 stock may be used. Other appropriately marked female stocks with multiple inverted X-chromosomes may also be used.

(4) *Control groups*—(i) *Concurrent controls.* Concurrent positive and negative (vehicle) controls shall be included in each experiment.

(ii) *Positive controls.* Examples of positive controls include ethyl methanesulfonate and N-nitroso-dimethylamine.

(iii) *Other positive controls.* Other positive control reference substances may be used.

(iv) *Negative controls.* Negative (vehicle) controls shall be included. The size of the negative (vehicle) control group shall be determined by the availability of appropriate laboratory historical control data.

(5) *Test chemicals*—(i) *Vehicle.* Test chemicals should be dissolved in water. Compounds which are insoluble in water may be dissolved or suspended in appropriate vehicles (e.g., a mixture of ethanol and Tween-60 or 80) and then diluted in water or saline prior to

administration. Dimethylsulfoxide should br avoided as a vehicle.

(ii) *Dose levels.* For the initial assessment of mutagenicity, it is sufficient to test a single dose of the test substance for screening purposes. This dose should be the maximum tolerated dose, or that which produces some indication of toxicity, or shall be the highest dose attainable. For dose-response purposes, at least three additional dose levels should be used.

(iii) *Route of administration.* Exposure may be oral, by injection or by exposure to gases or vapors. Feeding of the test compound may be done in sugar solution. When necessary, substances may be dissolved in 0.7 percent NaCl solution and injected into the thorax or abdomen.

(e) *Test performance*—(1) *Treatment and mating.* Wild-type males (3 to 5 days old) shall be treated with the test substance and mated individually to an appropriate number of virgin females from the Muller-5 stock or females from another appropriately marked (with multiply-inverted X-chromosomes) stock. The females shall be replaced with fresh virgins every 2 to 3 days to cover the entire germ cell cycle. The offspring of these females are scored for lethal effects corresponding to the effects on mature sperm, mid or late stage spermatids, early spermatids, spermatocytes and spermatogonia at the time of treatment.

(2) *F1 matings.* Heterozygous $F_1$ females from the above crosses shall be allowed to mate individually (i.e., one female per vial) with their brothers. In the $F_2$ generation, each culture shall be scored for the absence of wild-type males. If a culture appears to have arisen from an $F_1$ female carrying a lethal in the parental X-chromosome (i.e., no males with the treated chromosome are observed), daughters of that female with the same genotype shall be tested to ascertain if the lethality is repeated in the next generation.

(3) *Number of matings.* (i) The test should be designed with a predetermined sensitivity and power. The number of flies in each group should reflect these defined parameters. The spontaneous mutant frequency observed in the appropriate control group will strongly influence the number of treated chromosomes that must be analysed to detect substances which show mutation rates close to those of the controls.

(ii) Test results should be confirmed in a separate experiment.

(f) *Data and report*—(1) *Treatment of results.* Data shall be tabulated to show the number of chromosomes tested, the number of nonfertile males and the number of lethal chromosomes at each exposure concentration and for each mating period for each male treated. Numbers of clusters of different size per male shall be reported.

(2) *Statistical evaluation.* Data shall be evaluated by appropriate statistical techniques.

(3) *Interpretation of results.* (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of sex-lined recessive lethals. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test points.

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of sex-linked recessive lethals or a statistically significant and reproducible positive response at any one of the test points is considered non-mutagenic in this system.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(4) *Test evaluation.* (i) Positive results in the SLRL test in *D. melanogaster* indicate that under the test conditions the test agent causes mutations in germ cells of this insect.

(ii) Negative results indicate that under the test conditions the test substance is not mutagenic in *D. melanogaster.*

(5) *Test report.* In addition to the reporting recommendations as specified under 40 CFR Part 792, Subpart J the following specific information shall be reported.

(i) *Drosophila* stock used in the assay, age of insects, number of males treated, number of sterile males, number of $F_2$ cultures established, number of $F_2$ cultures without progeny.

(ii) Test chemical vehicle, treatment and sampling schedule, exposure levels, toxicity data, negative (vehicle) and positive controls, if appropriate.

(iii) Criteria for scoring lethals.

(iv) Number of chromosomes tested, number of chromosomes scored, number of chromosomes carrying a lethal mutation.

(v) Historical control data, if available.

(vi) Dose-response relationship, if applicable.

(g) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Sobels, F.H., Vogel, E. "The capacity of *Drosophila* for detecting relevant genetic damage," *Mutation Research* 41:95-106 (1976).

(2) Wurgler F.E., Sobels F.H., Vogel E. "*Drosophila* as assay system for detecting genetic changes," *Handbook of mutagenicity test procedures.* Eds. Kilbey, B.J., Legator, M., Nichols, W., Ramel, C., (Amsterdam: Elsevier/North Holland Biomedical Press, 1977) pp. 335-373.

[50 FR 39397, Sept. 27, 1985, as amended at 52 FR 19079, May 20, 1987]

### § 798.5300 Detection of gene mutations in somatic cells in culture.

(a) *Purpose.* Mammalian cell culture systems may be used to detect mutations induced by chemical substances. Widely used cell lines include L5178Y mouse lymphoma cells and the CHO and V-79 lines of Chinese hamster cells. In these cell lines the most commonly used systems measure mutation at the thymidine kinase (TK), hypoxanthine-guanine-phosphoribosyl transferase (HPRT) and $Na^+/K^+$ ATPase loci. The TK and HPRT mutational systems detect base pair mutations, frameshift mutations, and small deletions; the $Na^+/K^+$ ATPase system detects base pair mutations only.

(b) *Definitions.* (1) A forward mutation assay detects a gene mutation from the parental type to the mutant form which gives rise to a change in an enzymatic or functional protein.

(2) Base pair mutagens are agents which cause a base change in the DNA.

(3) Frameshift mutagens are agents which cause the addition or deletion of single or multiple base pairs in the DNA molecule.

(4) Phenotypic expression time is a period during which unaltered gene products are depleted from newly mutated cells.

(c) *Reference substances.* These may include, but need not be limited to, ethyl methanesulfonate, N-nitroso-dimethylamine, 2-acetylaminofluorene, 7,12-dimethylbenzanthracene or hycanthone.

(d) *Test method*—(1) *Principle.* Cells are exposed to test substance, both with and without metabolic activation, for a suitable period of time and subcultured to determine cytotoxicity and to allow phenotypic expression prior to mutant selection. Cells deficient in thymidine kinase (TK) due to the forward mutation $TK^+ \rightarrow TK^-$ are resistant to the cytotoxic effects of pyrimidine analogues such as bromodeoxyuridine (BrdU), fluorodeoxyuridine (FdU) or trifluorothymidine (TFT). The deficiency of the "salvage" enzyme thymidine kinase means that these antimetabolites are not incorporated into cellular nucleotides and the nucleotides needed for cellular metabolism are obtained solely from *de novo* synthesis. However, in the presence of thymidine kinase, BrdU, FdU or TFT are incorporated into the nucleotides, resulting in inhibition of cellular metabolism and cytotoxicity. Thus mutant cells are able to proliferate in the presence of BrdU, FdU or TFT whereas normal cells, which contain thymidine kinase, are not. Similarly cells deficient in HPRT are selected by resistance to 8-azaguanine (AG) or 6-thioguanine (TG) and cells with altered $Na^+/K^+$ ATPase are selected by resistance to ouabain.

(2) *Description.* Cells in suspension or monolayer culture are exposed to the test substance, both with and without metabolic activation, for a defined period of time. Cytotoxicity is determined by measuring the colony forming ability or growth rate of the cultures after the treatment period. The treated cultures are maintained in

growth medium for a sufficient period of time—characteristic of each selected locus—to allow near-optimal phenotypic expression of induced mutations. Mutant frequency is determined by seeding known numbers of cells in medium containing the selective agent to detect mutant cells, and in medium without selective agent to determine the cloning efficiency. After a suitable incubation time, cell colonies are counted. The number of mutant colonies in selective medium is adjusted by the number of colonies in nonselective medium to derive the mutant frequency.

(3) *Cells*—(i) *Type of cells used in the assay.* A variety of cell lines are available for use in this assay including subclones of L5178Y, CHO cells or V-79 cells. Cell types used in this assay should have a demonstrated sensitivity to chemical mutagens, a high cloning efficiency and a low spontaneous mutation frequency. Cells should be checked for *Mycoplasma* contamination and may be periodically checked for karyotype stability.

(ii) *Cell growth and maintenance.* Appropriate culture media and incubation conditions (culture vessels, $CO_2$ concentrations, temperature and humidity) shall be used.

(4) *Metabolic activation.* Cells shall be exposed to test substance both in the presence and absence of an appropriate metabolic activation system.

(5) *Control groups.* Positive and negative (untreated and/or vehicle) controls shall be included in each experiment. When metabolic activation is used, the positive control substance shall be known to require such activation.

(6) *Test chemicals*—(i) *Vehicle.* Test substances may be prepared in culture media or dissolved or suspended in appropriate vehicles prior to treatment of the cells. The final concentration of the vehicle shall not interfere with cell viability or growth rate. Treatment vessels should be chosen to ensure that there is no visible interaction, such as etching, between the solvent, the test chemical, and the vessel.

(ii) *Exposure concentrations.* (A) The test should be designed to have a predetermined sensitivity and power. The number of cells, cultures, and concentrations of test substance used should reflect these defined parameters. The number of cells per culture is based on the expected background mutant frequency; a general guide is to use a number which is 10 times the inverse of this frequency.

(B) Several concentrations (usually at least 4) of the test substance shall be used. Generally, these shall yield a concentration-related toxic effect. The highest concentration shall produce a low level of survival (approximately 10 percent), and the survival in the lowest concentration shall approximate the negative control. Cytotoxicity shall be determined after treatment with the test substance both in the presence and in the absence of an exogenous metabolic activation system. Relatively insoluble substances should be tested up to their limit of solubility under culture conditions. For freely-soluble nontoxic substances the highest concentration used should be determined on a case-by-case basis.

(e) *Test performance.* (1) Cells shall be exposed to the test substance both with and without exogenous metabolic activation. Exposure shall be for a suitable period of time, in most cases 1 to 5 hours is effective; exposure time may be extended over one or more cell cycles.

(2) At the end of the exposure period, cells shall be washed and cultured to determine viability and to allow for expression of the mutant phenotype.

(3) At the end of the expression period, which shall be sufficient to allow near optimal phenotypic expression of induced mutants, cells should be grown in medium with and without selective agent(s) for determination of number of mutants and cloning efficiency, respectively.

(4) Results shall be confirmed in an independent experiment. When appropriate, a single positive response should be confirmed by testing over a narrow range of concentrations.

(f) *Data and report*—(1) *Treatment of results.* Data shall be presented in tabular form. Individual colony counts for the treated and control groups shall be presented for both mutation induction and survival. Survival and cloning efficiencies shall be given as a percentage of the controls. Mutant frequency shall be expressed as number of mutants per number of surviving cells.

(2) *Statistical evaluation.* Data should be evaluated by appropriate statistical methods.

(3) *Interpretation of results.* (i) There are several criteria for determining a positive result, one of which is a statistically significant concentration-related increase in the mutant frequency. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test substance concentrations.

(ii) A test substance which does not produce either a statistically significant concentration-related increase in the mutant frequency or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(4) *Test evaluation.* (i) Positive results for an in vitro mammalian cell gene mutation test indicate that, under the test conditions, a substance induces gene mutations in the cultured mammalian cells used.

(ii) Negative results indicate that, under the test conditions, the test substance does not induce gene mutations in the cultured mammalian cells used.

(5) *Test report.* In addition to the reporting recommendations as specified under 40 CFR Part 792, Subpart J the following specific information shall be reported:

(i) Cell type used, number of cell cultures, methods used for maintenance of cell cultures.

(ii) Rationale for selection of concentrations and number of cultures.

(iii) Test conditions: composition of media, $CO_2$ concentration, concentration of test substance, vehicle, incubation temperature, incubation time, duration of treatment, cell density during treatment, type of metabolic activation system, positive and negative controls, length of expression period (including number of cells seeded and subculture and feeding schedules, if appropriate), selective agent(s).

(iv) Methods used to enumerate numbers of viable and mutant cells.

(v) Dose-response relationship, where possible.

(g) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Amacher, D.E., Paillet, S.C., Ray, V. "Point mutations at the thymidine kinase locus in L5178Y mouse lymphoma cells. I. Application to genetic toxicology testing," *Mutation Research*, 64:391-406 (1979).

(2) Amacher, D.E., Paillet, S.C., Turner, G.N., Ray, V.A. Salsburg, V.A. "Point mutations at the thymidine kinase locus in L5178Y mouse lymphoma cells. II. Test validation and interpretation," *Mutation Research*, 72:447-474 (1980).

(3) Bradley, M.O., Bhuyan B., Francis, M.C., Langenback, R., Peterson, A., Huberman, E. "Mutagenesis by chemical agents in V-79 Chinese hamster cells: a review and analysis of the literature: a report of the Gene-Tox Program," *Mutation Research*, 87:81-142 (1981).

(4) Clive, D., Johnson, K.O., Spector, J.F.S., Batson, A.G., Brown, M.M. "Validation and characterization of the L5178Y TK$^{+}/^{-}$ mouse lymphoma mutagen assay system," *Mutation Research*, 59:61-108 (1979).

(5) Clive, D., Spector, J.F.S. "Laboratory procedures for assessing specific locus mutations at the TK locus in cultured L5178Y mouse lymphoma cells," *Mutation Research*, 31:17-29 (1975).

(6) Hsie, A.W., Casciano, D.A., Couch, D.B., Krahn, D.F., O'Neill, J.P., Whitfield, B.L. "The use of Chinese hamster ovary cells to quantify specific locus mutation and to determine mutagenicity of chemicals: a report of the U.S. EPA's Gene-Tox Program," *Mutation Research*, 86:193-214 (1981).

[50 FR 39397, Sept. 27, 1985, as amended at 52 FR 19079, May 20, 1987]

### § 798.5375 In vitro mammalian cytogenetics.

(a) *Purpose.* The in vitro cytogenetics test is a mutagenicity test system for the detection of chromosomal aberrations in cultured mammalian cells. Chromosomal aberrations may be either structural or numerical. However, because cytogenetic assays are usually designed to analyse cells at their first post-treatment mitosis and numerical aberrations require at least one cell division to be visualized, this type of aberration is generally not observed in a routine cytogenetics assay. Structural aberrations may be of two types, chromosome or chromatid.

(b) *Definitions.* (1) Chromosome-type aberrations are changes which result from damage expressed in both sister chromatids at the same time.

(2) Chromatid-type aberrations are damage expressed as breakage of single chromatids or breakage and/or reunion between chromatids.

(c) *Reference substances.* Not applicable.

(d) *Test method*—(1) *Principle.* In vitro cytogenetics assays may employ cultures of established cell lines, cell strains or primary cell cultures. Cell cultures are exposed to the test substance both with and without metabolic activation. Following exposure of cell cultures to test substances, they are treated with a spindle inhibitor (e.g., colchicine or Colcemid®) to arrest cells in a metaphase-like stage of mitosis (c-metaphase). Cells are then harvested and chromosome preparations made. Preparations are stained and metaphase cells are analyzed for chromosomal aberrations.

(2) *Description.* Cell cultures are exposed to test compounds and harvested at various intervals after treatment. Prior to harvesting, cells are treated with a spindle inhibitor (e.g., colchicine or Colcemid®) to accumulate cells in c-metaphase. Chromosome preparations from cells are made, stained and scored for chromosomal aberrations.

(3) *Cells*—(i) *Type of cells used in the assay.* There are a variety of cell lines or primary cell cultures, including human cells, which may be used in the assay. Established cell lines and strains should be checked for *Mycoplasma* contamination and may be periodically checked for karyotype stability.

(ii) *Cell growth and maintenance.* Appropriate culture media, and incubation conditions (culture vessels $CO_2$ concentrations, temperature and humidity) shall be used.

(4) *Metabolic activation.* Cells shall be exposed to test substance both in the presence and absence of an appropriate metabolic activation system.

(5) *Control groups.* Positive and negative (untreated and/or vehicle) controls both with and without metabolic activation shall be included in each experiment. When metabolic activation is used, the positive control substance shall be known to require such activation.

(6) *Test chemicals*—(i) *Vehicle.* Test substances may be prepared in culture media or dissolved or suspended in appropriate vehicles prior to treatment of the cells. Final concentration of the vehicle shall not interfere with cell viability or growth rate. Treatment vessels should be chosen to ensure that there is no visible interaction, such as etching, between the solvent, the test chemical, and the vessel.

(ii) *Exposure concentrations.* Multiple concentrations of the test substance over a range adequate to define the response should be tested. Generally the highest test substance concentrations tested with and without metabolic activation should show evidence of cytotoxicity or reduced mitotic activity. Relatively insoluble substances should be tested up to the limit of solubility. For freely soluble nontoxic chemicals, the upper test chemical concentration should be determined on a case by case basis.

(e) *Test performance*—(1) *Established cell lines and strains.* Prior to use in the assay, cells should be generated from stock cultures, seeded in culture vessels at the appropriate density and incubated at 37 °C.

(2) *Human lymphocyte cultures.* Heparinized or acid-citrate-dextrose whole blood should be added to culture medium containing a mitogen, e.g., phytohemagglutinin (PHA) and incubated at 37 °C. White cells sedimented by gravity (buffy coat) or lymphocytes which have been purified on a density gradient may also be utilized.

(3) *Treatment with test substance.* For established cell lines and strains, cells in the exponential phase of growth shall be treated with test sub-

stances in the presence and absence of an exogenous metabolic activation system. Mitogen-stimulated human lymphocyte cultures may be treated with the test substance in a similar manner.

(4) *Number of cultures.* At least two independent cultures shall be used for each experimental point.

(5) *Culture harvest time.* (i) For established cell lines and strains, multiple harvest times are recommended. However, for screening purposes, a single harvest time may be appropriate. If the test chemical changes the cell cycle length, the fixation intervals should be changed accordingly. If a single harvest time is selected, supporting data for the harvest time should be presented in such a study.

(ii) For human lymphocyte cultures, the substance to be tested may be added to the cultures at various times after mitogen stimulation so that there is a single harvest time after the initiation of the cell culture. Alternatively, a single treatment may be followed by multiple harvest times. Harvest time should be extended for those chemicals which induce an apparent cell cycle delay. Because the population of human lymphocytes is only partially synchronized, a single treatment, at, or close to, the time when metaphase stages first appear in the culture will include cells in all phases of the division cycle. Therefore, a single harvest at the time of second mitosis may be carried out for screening purposes.

(iii) Cell cultures shall be treated with a spindle inhibitor, (e.g., colchicine or Colcemid®), 1 or 2 hours prior to harvesting. Each culture shall be harvested and processed separately for the preparation of chromosomes.

(6) *Chromosome preparation.* Chromosome preparation involves hypotonic treatment of the cells, fixation and staining.

(7) *Analysis.* Slides shall be coded before analysis. In human lymphocytes, only cells containing 46 centromeres shall be analyzed. In established cell lines and strains, only metaphases containing ±2 centromeres of the modal number shall be analyzed. Uniform criteria for scoring aberrations shall be used.

(8) *Confirmatory tests.* When appropriate, a single positive response shall be confirmed by testing over a narrow range of concentrations.

(f) *Data and report*—(1) *Treatment of results.* Data shall be presented in a tabular form. Different types of structural chromosomal aberrations shall be listed with their numbers and frequencies for experimental and control groups. Data should be evaluated by appropriate statistical methods. Gaps or achromatic lesions are recorded separately and not included in the total aberration frequency.

(2) *Statistical evaluation.* Data should be evaluated by appropriate statistical methods.

(3) *Interpretation of results.* (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of structural chromosomal aberrations. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test substance concentrations.

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of structural chromosomal aberrations or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(4) *Test evaluation.* (i) Positive results in the in vitro cytogenetics assay indicate that under the test conditions the test substance induces chromosomal aberrations in cultured mammalian somatic cells.

(ii) Negative results indicate that under the test conditions the test substance does not induce chromosomal aberrations in cultured mammalian somatic cells.

(5) *Test report.* In addition to the reporting recommendations as specified under 40 CFR Part 792, Subpart J the following specific information shall be reported:

(i) Cells used, density and passage number at time of treatment, number of cell cultures.

(ii) Methods used for maintenance of cell cultures including medium, temperature and CO$_2$ concentration.

(iii) Test chemical vehicle, concentration and rationale for the selection of the concentrations used in the assay, duration of treatment.

(iv) Details of both the protocol used to prepare the metabolic activation system and of its use in the assay.

(v) Identity of spindle inhibitor, its concentration and duration of treatment.

(vi) Date of cell harvest.

(vii) Positive and negative controls.

(viii) Methods used for preparation of slides for microscopic examination.

(ix) Number of metaphases analysed.

(x) Mitotic index where applicable.

(xi) Criteria for scoring aberrations.

(xii) Type and number of aberrations, given separately for each treated and control culture, total number of aberrations per group; frequency distribution of number of chromosomes in established cell lines and strains.

(xiii) Dose-response relationship, if applicable.

(g) *References.* For additional background information on this test guideline the following references should be consulted.

(1) Ames, B.N., McCann, J., Yamasaki, E. "Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test," *Mutation Research*, 31:347-364 (1975).

(2) Evans, H.J. "Cytological methods for detecting chemical mutagens," *Chemical mutagens, principles and methods for their detection*, Vol. 4, Ed. A. Hollaender (New York, London: Plenum Press, 1976) pp. 1-29.

(3) Howard, P.N., Bloom, A.D., Krooth, R.S. "Chromosomal aberrations induced by N-methyl-N'-nitro-N-nitrosoguanidine in mammalian cells," *In Vitro* 7:359-365 (1972).

(4) Ishidate, M. Jr., Odashima, S. "Chromosome tests with 134 compounds on Chinese hamster cells in vitro: A screening for chemical carcinogens," *Mutation Research*, 48:337-354 (1975).

(5) Preston, R.J., Au, W., Bender, M.A., Brewen, J.G., Carrano, A.V., Heddle, J.A., McFee, A.F., Wolff, S., Wassom, J.S., "Mammalian in vivo and in vitro cytogenetic assays: A report of the Gene-tox Program," *Mutation Research*, 87:143-188 (1981).

[50 FR 39397, Sept. 27, 1985, as amended at 52 FR 19079, May 20, 1987]

### § 798.5385 In vivo mammalian bone marrow cytogenetics tests: Chromosomal analysis.

(a) *Purpose.* The *in vivo* bone marrow cytogenetic test is a mutagenicity test for the detection of structural chromosomal aberrations. Chromosomal aberrations are generally evaluated in first post-treatment mitoses. With the majority of chemical mutagens, induced aberrations are of the chromatid type but chromosome type aberrations also occur.

(b) *Definitions.* (1) Chromosome-type aberrations are changes which result from damage expressed in both sister chromatids at the same time.

(2) Chromatid-type aberrations are damage expressed as breakage of single chromatids or breakage and/or reunion between chromatids.

(c) *Reference substances.* Not applicable.

(d) *Test method*—(1) *Principle.* Animals are exposed to test chemicals by appropriate routes and are sacrificed at sequential intervals. Chromosome preparations are made from bone marrow cells. The stained preparations are examined and metaphase cells are scored for chromosomal aberrations.

(2) *Description.* The method employs bone marrow of laboratory rodents which have been exposed to test chemicals. Prior to sacrifice, animals are further treated with a spindle inhibitor, (e.g., colchicine or Colcemid ®) to arrest the cells in c-metaphase. Chromosome preparations from the cells are stained and scored for chromosomal aberrations.

(3) *Animal selection*—(i) *Species and strain.* Any appropriate mammalian species may be used. Examples of commonly used rodent species are rats, mice, and hamsters.

(ii) *Age.* Healthy young adult animals shall be used.

(iii) *Number and sex.* At least five female and five male animals per experimental and control group shall be used. Thus, 10 animals would be sacrificed per time per group treated with the test compound if several test times after treatment are included in the experimental schedule. The use of a single sex or smaller number of animals should be justified.

(iv) *Assignment to groups.* Animals shall be randomized and assigned to treatment and control groups.

(4) *Control groups*—(1) *Concurrent controls.* (i) Concurrent positive and negative (vehicle) controls shall be included in the assay.

(ii) *Positive controls.* A single dose positive control showing a significant response at any one time point is adequate. A compound known to produce chromosomal aberrations in vivo shall be employed as the positive control.

(5) *Test chemicals*—(i) *Vehicle.* When possible, test chemicals shall be dissolved in isotonic saline or distilled water. Water insoluble chemicals may be dissolved or suspended in appropriate vehicles. The vehicles used shall neither interfere with the test chemical nor produce toxic effects. Fresh preparations of the test compound should be employed.

(ii) *Dose levels.* For an initial assessment, one dose of the test substance may be used, the dose being the maximum tolerated dose (to a maximum of 5,000 mg/kg) or that producing some indication of cytotoxicity (e.g., partial inhibition of mitosis) or shall be the highest dose attainable (to a maximum of 5,000 mg/kg). Additional dose levels may be used. For determination of dose-response, at least three dose levels should be used.

(iii) *Route of administration.* The usual routes are oral or by intraperitoneal injection. Other routes may be appropriate.

(iv) *Treatment schedule.* In general, test substances should be administered once only. However, based on toxicological information a repeated treatment schedule may be employed.

(e) *Test performance*—(1) *Generally the test may be performed in two assays.* (i) Animals should be treated with the test substance once at the selected dose(s). Samples should be taken at three times after treatment. For rodents, the central sampling interval is 24 hours. Since cell cycle kinetics can be influenced by the test substance, one earlier and one later sampling interval adequately spaced within the range of 6 to 48 hours shall be applied. Where the additional dose levels are tested in a subsequent experiment, samples shall be taken at the predetermined most sensitive interval or, if this is not established, at the central sampling time. If the most sensitive interval is known and documented with data, only this one time point shall be sampled.

(ii) If a repeated treatment schedule is used at the selected dose(s), samples shall be taken 6 and 24 hours after the last treatment; other sampling times may be used if justified. Where the additional dose levels are tested in a subsequent experiment, samples shall be taken at the predetermined most sensitive interval or, if this is not established, at 6 hours after the last treatment.

(2) *Administration of spindle inhibitor.* Prior to sacrifice, animals shall be injected IP with an appropriate dose of a spindle inhibitor (e.g., colchicine or Colcemid®) to arrest cells in c-metaphase.

(3) *Preparation of slides.* Immediately after sacrifice, the bone marrow shall be obtained, exposed to hypotonic solution, and fixed. The cells shall then be spread on slides and stained. Chromosome preparations shall be made following standard procedures.

(4) *Analysis.* The number of cells to be analyzed per animal should be based upon the number of animals used, the negative control frequency, the predetermined sensitivity, and the power chosen for the test. Slides shall be coded before microscopic analysis.

(f) *Data and report*—(1) *Treatment of results.* Data should be presented in tabular form for both cells and animals. Different types of structural chromosomal aberrations should be listed with their numbers and a mean frequency per cell for each animal in all treated and control groups. Gaps (achromatic lesions) should be recorded separately and not included in the total abberration frequency. Differences among animals within each group should be considered before making comparisons between treated and control groups.

(2) *Statistical evaluation.* Data should be evaluated by appropriate statistical methods.

(3) *Interpretation of results.* (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of structual chromosomal aberrations or abnormal metaphase figures. Another criterion may be based upon detection of a reproducible and statistically significant positive response for a least one of the test points.

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of chromosomal aberrations or abnormal metaphase figures or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(4) *Test evaluation.* (i) Positive results in the *in vivo* bone marrow cytogenetics assay indicate that under the test conditions the test substance induces chromosomal aberrations in the bone marrow of the test species.

(ii) Negative results indicate that under the test conditions, the test substance does not induce chromosomal aberrations in the bone marrow of the test species.

(5) *Test report.* In addition to the reporting recommendations as specified under 40 CFR Part 792, Subpart J the following specific information shall be reported:

(i) Species, strain, age, weight, number and sex of animals in each treatment and control group.

(ii) Test chemical vehicle, dose levels used, rationale for dose selection.

(iii) Route of administration, treatment and sampling schedules, toxicity data, negative and positive controls.

(iv) Identity of spindle-inhibitor, its concentration and duration of treatment.

(v) Details of the protocol used for chromosome preparation, number of cells scored per animal, type and number of aberrations given separately for each treated and control animal.

(vi) Mitotic index, where applicable.

(vii) Criteria for scoring aberrations.

(viii) Number and frequency of aberrant cells per animal in each treatment and control groups.

(ix) Total number of aberrations per group.

(x) Number of cells with aberrations per group.

(xi) Dose-response relationship, if applicable.

(g) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Adler, I.D., Ramarao, G., Epstein, S.S. "In vivo cytogenetic effects of trimethyl-phosphate and of TEPA on bone marrow cells of male rats," *Mutation Research,* 13:263-273 (1971).

(2) Evans, H.J. "Cytological methods for detecting chemical mutagens," *Chemical Mutagens: Principles and Methods for Their Detection,* Vol. 4. Ed. A. Hollaender (New York and London: Plenum Press, 1976) pp. 1-29.

(3) Kilian, J.D., Moreland, F.E. Benge, M.C., Legator, M.S., Whorton, E.B. Jr. "A collaborative study to measure intralaboratory variation with the in vivo bone morrow metaphase procedure," *Handbook of mutagenicity test procedures.* Eds. Kilby, B.J., Legator, M. Nichols, C., Ramel, D., (Amsterdam: Elsevier/North Holland Biomedical Press, 1977) 243-260.

(4) Preston, J.R., Au, W., Bender, M.A., Brewen, J.G., Carrano, A.V. Heddle, J.A., McFee, A.F., Wolff, S., Wassom, J. "Mammalian in vivo and vitro cytogenetics assays: Report of the Gene-Tox Program," *Mutation Research, 87:143-188 (1981).*

[50 FR 39397, Sept. 27, 1985, as amended at 52 FR 19080, May 20, 1987]

### § 798.5395 In vivo mammalian bone marrow cytogenetics tests: Micronucleus assay.

(a) *Purpose.* The micronucleus test is a mammalian *in vivo* test which detects damage of the chromosomes or mitotic apparatus by chemicals. Polychromatic erythrocytes in the bone marrow of rodents are used in this assay. When the erythroblast develops into an erythrocyte the main nucleus is extruded and may leave a micronucleus in the cytoplasm. The visualization of micronuclei is facilitated in these cells because they lack a nucleus. Micronuclei form under normal conditions. The assay is based on an increase in the frequency of micronucleated polychromatic erythrocytes in bone marrow of treated animals.

(b) *Definition.* Micronuclei are small particles consisting of acentric fragments of chromosomes or entire chromosomes, which lag behind at anaphase of cell division. After telophase, these fragments may not be included in the nuclei of daughter cells and form single or multiple micronuclei in the cytoplasm.

(c) *Reference substances.* Not applicable.

(d) *Test method*—(1) *Principle.* (i) Animals are exposed to test substance by an appropriate route. They are sacrificed, the bone marrow extracted and smear preparations made and stained. Polychromatic erythrocytes are scored for micronuclei under the microscope.

(ii) Micronuclei may also be detected in other test systems:

(A) Tissue culture.

(B) Plants.

(C) Blood smears.

(D) Fetal tissues.

(E) Meiotic cells.

(F) Hepatic cells.

(iii) The present guideline is based on the mammalian bone marrow assay.

(2) *Description.* The method employs bone marrow of laboratory mammals which are exposed to test substances.

(3) *Animal selection*—(i) *Species and strain.* Mice are recommended. However, any appropriate mammalian species may be used.

(ii) *Age.* Young adult animals shall be used.

(iii) *Number and sex.* At least five female and five male animals per experimental and control group shall be used. Thus, 10 animals would be sacrificed per time per group if several test times after treatment were included in the experimental schedule. The use of a single sex or a smaller number of animals should be justified.

(iv) *Assignment to groups.* Animals shall be randomized and assigned to treatment and control groups.

(4) *Control groups*—(i) *Concurrent controls.* Concurrent positive and negative (vehicle) controls shall be included in each assay.

(ii) *Positive controls.* A compound known to produce micronuclei *in vivo* shall be employed as the positive control.

(5) *Test chemicals*—(i) *Vehicle.* When appropriate for the route of administration, solid and liquid test substances should be dissolved or suspended in distilled water or isotonic saline. Water insoluble chemicals may be dissolved or suspended in appropriate vehicles. The vehicle used shall neither interfere with the test compound nor produce toxic effects. Fresh preparations of the test compound should be employed.

(ii) *Dose levels.* For an initial assessment, one dose of the test substance may be used, the dose being the maximum tolerated dose (to a maximum of 5,000 mg/kg) or that producing some indication of cytotoxicity, e.g., a change in the ratio of polychromatic to normochromatic erythrocytes. Additional dose levels may be used. For determination of dose response, at least three dose levels shall be used.

(iii) *Route of administration.* The usual routes of administration are IP or oral. Other routes may be appropriate.

(iv) *Treatment schedule.* Test substances should generally be administered only once. However, based upon toxicological information a repeated treatment schedule may be employed.

(e) *Test performance*—(1) *Treatment and sampling times.* (i) Animals shall be treated with the test substance once at the highest tolerated dose. Sampling times should coincide with the maximum responses of the assay which varies with the test substance. Therefore, using the highest dose, bone marrow samples should be taken at least three times, starting not earlier than 12 hours after treatment, with appropriate intervals following the first sample but not extending beyond 72 hours. When other doses are used sampling shall be at the maximum sensitive period, or, if that is not known, approximately 24 hours after treatment. Other appropriate sampling times may be used in addition. If the most sensitive interval is known and documented with data, only this one time point need be sampled.

(ii) If a repeated treatment schedule is used, samples shall be taken at least three times, starting not earlier than 12 hours after the last treatment and at appropriate intervals following the first sample, but not extending beyond 72 hours.

(iii) Bone marrow shall be obtained immediately after sacrifice. Cells shall be prepared, put on slides, spread as a smear and stained.

(2) *Analysis*. Slides shall be coded before microscopic analysis. At least 1,000 polychromatic erythrocytes per animal shall be scored for the incidence of micronuclei. The ratio of polychromatic to normochromatic erythrocytes should be determined for each animal by counting a total of 200 erythrocytes. To ensure consistency with OECD and other guidelines, 1,000 polychromatic erythrocytes are recommended. Additional information may be obtained by scoring normochromatic erythrocytes for micronuclei.

(f) *Data and report*—(1) *Treatment of results*. Criteria for scoring micronuclei shall be given. Individual data shall be presented in a tabular form including positive and negative (vehicle) controls and experimental groups. The number of polychromatic erythrocytes scored, the number of micronucleated polychromatic erythrocytes, the percentage of micronucleated cells, the number of micronucleated normochromatic erythrocytes, and, if applicable, the percentage of micronucleated erythrocytes and the ratio of normochromatic to polychromatic erythrocytes shall be listed separately for each experimental and control animal. Absolute numbers shall be included if percentages are reported.

(2) *Statistical evaluation*. Data should be evaluated by appropriate statistical methods.

(3) *Interpretation of results*. (i) There are several criteria for determining a positive response, one of which is a statistically significant dose-related increase in the number of micronucleated polychromatic erythrocytes. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test substance concentrations.

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of micronucleated polychromatic erythrocytes or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(4) *Test evaluation*. (i) The results of the micronucleus test provide information on the ability of a chemical to induce micronuclei in polychromatic erythrocytes of the test species under the conditions of the test. This damage may have been the result of chromosomal damage or damage to the mitotic apparatus.

(ii) Negative results indicate that under the test conditions the test substance does not produce micronuclei in the bone marrow of the test species.

(5) *Test report*. In addition to the reporting recommendations as specified under 40 CFR Part 792, Subpart J, the following specific information shall be reported:

(i) Species, strain, age, weight, number and sex of animals in each treatment and control group.

(ii) Test chemical vehicle, dose levels used, rationale for dose selection.

(iii) Rationale for and description of treatment and sampling schedules, toxicity data, negative and positive controls.

(iv) Details of the protocol used for slide preparation.

(v) Criteria for identifying micronucleated erythrocytes.

(vi) Dose-response relationship, if applicable.

(g) *References*. For additional background information on this test guideline the following references should be consulted:

(1) Cihak, R. "Evaluation of benzidine by the micronucleus test," *Mutation Research*, 67: 383-384 (1979).

(2) Cole, R.J., Taylor, N., Cole, J., Arlett, C.F. "Short-term tests for transplacentally active carcinogens. 1. Micronucleus formation in fetal and maternal mouse erythroblasts," *Mutation Research*, 80: 141-157 (1981).

(3) Kliesch, U., Danford, N., Adler, I.D. "Micronucleus test and bone-marrow chromosome analysis. A comparison of 2 methods in vivo for evaluating chemically induced chromosomal alterations," *Mutation Research*, 80: 321-332 (1981).

(4) Matter, B., Schmid, W. "Trenimon-induced chromosomal damage in bone-marrow cells of six mammalian species, evaluated by the micronucleus test," *Mutation Research*, 12: 417-425 (1971).

(5) Schmid, W. "The micronucleus test," *Mutation Research, 31:9-15 (1975).*

(6) Schmid, W. "The micronucleus test for cytogenetic analysis," *Chemical Mutagens, Principles and Methods for their Detection.* Vol. 4 Hollaender A, (Ed. A ed. (New York and London: Plenum Press, (1976) pp. 31-53.

(7) Heddle, J.A., Hite, M., Kurkhart, B., Mavournin, K., MacGregor, J.T., Newell, G.W., Salamone, M.F. "The induction of micronuclei as a measure of genotoxicity. A report of the U.S. Environmental Protection Agency Gene-Tox Program," *Mutation Research*, 123: 61-118 (1983).

[50 FR 39397, Sept. 27, 1985, as amended at 52 FR 19080, May 20, 1987; 52 FR 26150, July 13, 1987; 52 FR 34654, Sept. 14, 1987]

## § 798.5450 Rodent dominant lethal assay.

(a) *Purpose.* Dominant lethal (DL) effects cause embryonic or fetal death. Induction of a dominant lethal event after exposure to a chemical substance indicates that the substance has affected germinal tissue of the test species. Dominant lethals are generally accepted to be the result of chromosomal damage (structural and numerical anomalies) but gene mutations and toxic effects cannot be excluded.

(b) *Definition.* A dominant lethal mutation is one occurring in a germ cell which does not cause dysfunction of the gamete, but which is lethal to the fertilized egg or developing embryo.

(c) *Reference substances.* These may include, but need not be limited to, triethylenemelamine, cyclophosphamide or ethyl methanesulfonate.

(d) *Test method*—(1) *Principle.* Generally, male animals are exposed to the test substance and mated to untreated virgin females. The various germ cell stages can be tested separately by the use of sequential mating intervals. The females are sacrificed after an appropriate period of time and the contents of the uteri are examined to determine the numbers of implants and live and dead embryos. The calculation of the dominant lethal effect is based on comparison of the live implants per female in the treated group to the live implants per female in the control group. The increase of dead implants per female in the treated group over the dead implants per female in the control group reflects the post-implantation loss. The post-implantation loss is calculated by determining the ratio of dead to total implants from the treated group compared to the ratio of dead to total implants from the control group. Preimplantation loss can be estimated on the basis of corpora lutea counts or by comparing the total implants per female in treated and control groups.

(2) *Description.* (i) Several treatment schedules are available. The most widely used requires single administration of the test substance. Other treatment schedules, such as treatment on five consecutive days, may be used if justified by the investigator.

(ii) Individual males are mated sequentially to virgin females at appropriate intervals. The number of matings following treatment is governed by the treatment schedule and should ensure that germ cell maturation is adequately covered. Females are sacrificed in the second half of pregnancy and the uterine contents examined to determine the total number of implants and the number of live and dead embryos.

(3) *Animal selection*—(i) *Species.* Rats or mice are generally used as the test species. Strains with low background dominant lethality, high pregnancy frequency and high implant numbers are recommended.

(ii) *Age.* Healthy, sexually mature animals shall be used.

(iii) *Number.* An adequate number of animals shall be used taking into account the spontaneous variation of the biological characteristics being evaluated. The number chosen should be based on the predetermined sensitivity of detection and power of significance. For example, in a typical experiment, the number of males in each group shall be sufficient to provide between 30 and 50 pregnant females per mating interval.

(iv) *Assignment to groups.* Animals shall be randomized and assigned to treatment and control groups.

(4) *Control groups*—(i) *Concurrent controls.* Generally concurrent positive and negative (vehicle) controls shall be included in each experiment. When acceptable positive control results are available from experiments conducted recently (within the last 12 months) in the same laboratory these results can be used instead of a concurrent positive control.

(ii) *Positive controls.* Positive control substances shall be used at a dose which demonstrates the test sensitivity.

(5) *Test chemicals*—(i) *Vehicle.* When possible, test substances shall be dissolved or suspended in isotonic saline or distilled water. Water-insoluble chemicals may be dissolved or suspended in appropriate vehicles. The vehicle used shall neither interfere with the test chemical nor produce toxic effects. Fresh preparations of the test chemical should be employed.

(ii) *Dose levels.* Normally, three dose levels shall be used. The highest dose shall produce signs of toxicity (e.g., slightly reduced fertility and slightly reduced body weight). However, in an initial assessment of dominant lethality a single high dose may be sufficient. Nontoxic substances shall be tested at 5g/kg or, if this is not practicable, then as the highest dose attainable.

(iii) *Route of administration.* The usual routes of administration are oral or by IP injection. Other routes may be appropriate.

(e) *Test performance.* (1) Individual males are mated sequentially at appropriate predetermined intervals to one or two virgin females. Females should be left with the males for at least the duration of one estrus cycle or alternatively until mating has occurred as determined by the presence of sperm in the vagina or by the presence of a vaginal plug.

(2) The number of matings following treatment should be governed by the treatment schedule and should ensure that germ cell maturation is adequately covered.

(3) Females should be sacrificed in the second half of pregnancy and uterine contents examined to determine the number of implants and live and dead embryos. The ovaries may be examined to determine the number of corpora lutea.

(f) *Data and report*—(1) *Treatment of results.* Data shall be tabulated to show the number of males, the number of pregnant females, and the number of nonpregnant females. Results of each mating, including the identity of each male and female, shall be reported individually. For each female, the dose level and week of mating and the frequencies of live implants and of dead implants shall be enumerated. If the data are recorded as early and late deaths, the tables shall make that clear. If preimplantation loss is estimated, it shall be reported. Preimplantation loss can be calculated as the difference between the number of corpora lutea and the number of implants or as a reduction in the average number of implants per female in comparison with control matings.

(2) *Statistical evaluation.* Data shall be evaluated by appropriate statistical methods. Differences among animals within the control and treatment groups shall be considered before making comparisons between treated and control groups.

(3) *Interpretation of results.* (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of dominant lethals. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test points.

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of dominant lethals or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(4) *Test evaluation.* (i) A positive DL assay suggests that under the test conditions the test substance may be genotoxic in the germ cells of the treated sex of the test species.

(ii) A negative result suggests that under the conditions of the test the test substance may not be genotoxic in the germ cells of the treated sex of the test species.

(5) *Test Report.* In addition to the reporting recommendations as specified under 40 CFR Part 792, Subpart J the following specific information shall be reported:

(i) Species, strain, age and weights of animals used, number of animals of each sex in experimental and control groups.

(ii) Test substance, vehicle used, dose levels and rationale for dosage selection, negative (vehicle) and positive controls, experimental observations, including signs of toxicity.

(iii) Route and duration of exposure.

(iv) Mating schedule.

(v) Methods used to determine that mating has occurred (where applicable).

(vi) Criteria for scoring dominant lethals including the number of early and late embryonic deaths.

(vii) Dose-response relationship, if applicable.

(g) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Brewen, J.G., Payne, H.S., Jones, K.P., Preston, R.J. "Studies on chemically induced dominant lethality. I. The cytogenetic basis of MMS-induced dominant lethality in post-meiotic germ cells" *Mutation Research,* 33:239-250 (1975).

(2) Ehling, U.H., Machemer, L., Buselmaier, E., Dycka, D., Frohberg, H., Kratochvilova, J., Lang, R., Lorke, D., Muller, D., Pheh, J., Rohrborn, G., Roll, R., Schulze-Schencking, M., Wiemann, H. "Standard protocol for the dominant lethal test on male mice. Set up by the Work Group "Dominant lethal mutations of the ad hoc Committee Chemogenetics," *Archives of Toxicology,* 39:173-185 (1978).

[50 FR 39397, Sept. 27, 1985, as amended at 52 FR 19081, May 20, 1987]

### § 798.5460 Rodent heritable translocation assays.

(a) *Purpose.* This test detects transmitted chromosomal damage which manifests as balanced reciprocal translocations in progeny descended from parental males treated with chemical mutagens.

(b) *Definitions.* (1) A heritable translocation is one in which distal segments of nonhomologous chromosomes are involved in a reciprocal exchange.

(2) Diakinesis and metaphase I are stages of meiotic prophase scored cytologically for the presence of multivalent chromosome association characteristic of translocation carriers.

(c) *Reference substances.* Not applicable.

(d) *Test method*—(1) *Principle.* When a balanced reciprocal translocation is induced in a parental male germ cell, the resulting progeny is translocation heterozygote.

(i) *Basis for fertility screening.* Male translocation heterozygotes may be completely sterile. This class consists of two types of translocations:

(A) Translocations between non-homologous chromosomes in which at least one of the breaks occurs close to one end of a chromosome.

(B) Those that carry multiple translocations. The majority of male translocation heterozygotes are semisterile—they carry one or (rarely) two translocations. The degree of semisterility is dependent upon the proportions of balanced and unbalanced (duplication-deficiency) gametes produced in the ejaculate as a function of meiotic segregation. Balanced and unbalanced sperm are equally capable of fertilizing an egg. Balanced sperm lead to viable progeny. Unbalanced sperm result in early embryonic lethality.

(ii) *Basis for cytological screening.* The great majority of male translocation heterozygotes can be identified cytologically through analysis of diakinesis metaphase I spermatocytes. Translocation heterozygotes are characterized by the presence of multivalent chromosome association such as a ring or chain of four chromosomes held together by chiasmata in paired homologous regions. Some translocation carriers can be identified by the presence of extra long and/or extra short chromosomes in spermatogonial and somatic cell metaphase preparations.

(2) *Description.* Essentially, two methods have been used to screen for translocation heterozygosity; one method uses a mating sequence to identify sterile and semisterile males followed by cytological examination of suspect male individuals; the other method deletes the mating sequence altogether and all $F_1$ male progeny are examined cytologically for presence of translocation. In the former approach, the mating sequence serves as a screen which eliminates most fully fertile animals for cytological confirmation as translocation heterozygotes.

(3) *Animal selection*—(i) *Species.* The mouse is the species generally used, and is recommended.

(ii) *Age.* Healthy sexually mature animals shall be used.

(iii) *Number.* (A) The number of male animals necessary is determined by the following factors:

(*1*) The use of either historical or concurrent controls.

(*2*) The power of the test.

(*3*) The minimal rate of induction required.

(*4*) Whether positive controls are used.

(*5*) The level of significance desired.

(B) [Reserved]

(iv) *Assignment to groups.* Animals shall be randomized and assigned to treatment and control groups.

(4) *Control groups*—(i) *Concurrent controls.* No concurrent positive or negative (vehicle) controls are recommended as routine parts of the heritable translocation assay. However, investigators not experienced in performing translocation testing shall include a substance known to produce translocations in the assay as a positive control reference chemical.

(ii) *Historical controls.* At the present time, historical control data must be used in tests for significance. When statistically reliable historical controls are not available, negative (vehicle) controls shall be used.

(5) *Test chemicals*—(i) *Vehicle.* When appropriate for the route of administration, solid and liquid test substances should be dissolved or suspended in distilled water or isotonic saline. Water-insoluble chemicals may be dissolved or suspended in appropriate vehicles. The vehicle used shall neither interfere with the test chemical nor produce toxic effects. Fresh preparations of the test chemical should be employed.

(ii) *Dose levels.* At least two dose levels shall be used. The highest dose level shall result in toxic effects (which shall not produce an incidence of fatalities which would prevent a meaningful evaluation) or shall be the highest dose attainable or 5g/kg body weight.

(iii) *Route of administration.* Acceptable routes of administration include oral, inhalation, admixture with food or water, and IP or IV injection.

(e) *Test performance*—(1) *Treatment and mating.* The animals shall be dosed with the test substances 7 days per week over a period of 35 days. After treatment, each male shall be caged with 2 untreated females for a period of 1 week. At the end of 1 week, females shall be separated from males and caged individually. When females give birth, the day of birth, litter size, and sex of progeny shall be recorded. All male progeny should be weaned, and all female progeny should be discarded.

(2) *Testing for translocation heterozygosity.* When males are sexually mature, testing for translocation heterozygosity shall begin. One of two methods shall be used; the first method involves mating, determining those $F_1$ progeny which are sterile or semisterile and subsequent cytological analysis of suspect progeny; the other method does not involve mating and determining sterility or semisterility; all progeny are examined cytologically.

(i) *Determination of sterility or semisterility*—(A) *Conventional method.* Females are mated, usually three females for each male, and each female is killed at midpregnancy. Living and dead implantations are counted. Criteria for determining normal and semisterile males are usually established for each new strain because the number of dead implantations varies considerably among strains.

(B) *Sequential method.* Males to be tested are caged individually with females and the majority of the presumably normal males are identified on the basis of a predetermined size of 1 or 2 litters. Breeding pens are examined daily on weekdays beginning 18 days after pairing. Young are discarded immediately after they are scored. Males that sire a litter whose size is the same as or greater than the minimum set for a translocation-free condition are discarded with their litter. If the litter size is smaller than the predetermined number, a second litter is produced with the same rule applying. Males that cannot be classified as normal after production of a second litter are tested further by the conventional method or by cytological confirmation of translocation.

(ii) *Cytological analysis.* For cytological analysis of suspected semisteriles, the air-drying technique is used. Observation of at least 2 diakinesis-metaphase 1 cells with multivalent association constitutes the required evidence for the presence of a translocation. Sterile males are examined by one of two methods, those with testes of normal size and sperm in the epididymis are examined by the same techniques used for semisteriles. Animals with small testes are examined by squash preparations or, alternatively, by examination of mitotic metaphase preparations. If squash preparations do not yield diakinesis-metaphase 1 cells, analysis of spermatogonia or bone marrow for the presence of unusually long or short chromosomes should be performed.

(f) *Data and report*—(1) *Treatment of results.* (i) Data shall be presented in tabular form and shall include the number of animals at risk, the germ cell stage treated, the number of partial steriles and semisteriles (if the fertility test is used), the number of cytogenetically confirmed translocation heterozygotes (if the fertility test is used, report the number of confirmed steriles and confirmed partial steriles), the translocation rate, and either the standard error of the rate or the upper 95 percent confidence limit on the rate.

(ii) These data shall be presented for both treated and control groups. Historical or concurrent controls shall be specified, as well as the randomization procedure used for concurrent controls.

(2) *Statistical evaluation.* Data shall be evaluated by appropriate statistical methods.

(3) *Interpretation of results.* (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of heritable translocations. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test points.

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of heritable translocations or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(4) *Test evaluation.* (i) Positive results in the heritable translocation assay indicate that under the test conditions the test substance causes heritable chromosomal damage in the test species.

(ii) Negative results indicate that under the test conditions the test substance does not cause heritable chromosomal damage in the test species.

(5) *Test report.* In addition to the reporting recommendations as specified under 40 CFR Part 792, Subpart J, the following specific information shall be reported:

(i) Species, strain, age, weight and number of animals of each sex in each group.

(ii) Test chemical vehicle, route and schedule of administration, toxicity data.

(iii) Dosing regimen, doses tested and rationale for dosage selection.

(iv) Mating schedule, number of females mated to each male.

(v) The use of historical or concurrent controls.

(vi) Screening procedure including the decision criteria used and the method by which they were determined.

(vii) Dose-response relationship, if applicable.

(g) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Generoso, W.M., Bishop, J.B., Goslee, D.G., Newell, G.W., Sheu, G-J, von Halle, E. "Heritable translocation test in mice," *Mutation Research*, 76:191-215 (1980).

(2) [Reserved]

[50 FR 39397, Sept. 27, 1985, as amended at 52 FR 19081, May 20, 1987]

### § 798.5500 Differential growth inhibition of repair proficient and repair deficient bacteria: "Bacterial DNA damage or repair tests."

(a) *Purpose.* Bacterial DNA damage or repair tests measure DNA damage which is expressed as differential cell killing or growth inhibition of repair deficient bacteria in a set of repair proficient and deficient strains. These tests do not measure mutagenic events *per se*. They are used as an indication of the interaction of a chemical with genetic material implying the potential for genotoxicity.

(b) *Definition.* Test for differential growth inhibition of repair proficient and repair deficient bacteria measure differences in chemically induced cell killing between wild-type strains with full repair capacity and mutant strains deficient in one or more of the enzymes which govern repair of damaged DNA.

(c) *Reference substances.* These may include, but need not be limited to, chloramphenicol or methyl methanesulfonate.

(d) *Test method*—(1) *Principle.* The tests detect agents that interact with cellular DNA to produce growth inhibition or killing. This interaction is recognized by specific cellular repair systems. The assays are based upon the use of paired bacterial strains that differ by the presence of absence of specific DNA repair genes. The response is expressed in the preferential inhibition of growth or the preferential killing of the DNA repair deficient strain since it is incapable of removing certain chemical lesions from its DNA.

(2) *Description.* Several methods for performing the test have been described. Those described here are:

(i) Tests performed on solid medium (diffusion tests).

(ii) Tests performed in liquid culture (suspension tests).

(3) *Strain selection*—(i) *Designation.* At the present time, *Escherichia coli polA* (W3110/p3478) or *Bacillus subtilis rec* (H17, M45) pairs are recommended. Other pairs may be utilized when appropriate.

(ii) *Preparation and storage.* Stock culture preparation and storage, growth requirements, method of strain identification and demonstration of appropriate phenotypic requirements should be performed using good microbiological techniques and should be documented.

(4) *Bacterial growth.* Good microbiological techniques should be used to grow fresh cultures of bacteria. The phase of growth and cell density should be documented and should be adequate for the experimental design.

(5) *Metabolic activation.* Bacteria should be exposed to the test substance both in the presence and absence of an appropriate metabolic activation system. The most commonly used system is a cofactor supplemented postmitochondrial fraction prepared from the livers of rodents treated with enzyme inducing agents. The use of other species, tissues or techniques may also be appropriate.

(6) *Control groups*—(i) *Concurrent controls.* Concurrent positive, negative, and vehicle controls should be included in each assay.

(ii) *Negative controls.* The negative control should show nonpreferential growth inhibition (i.e., should affect both strains equally). Chloramphenicol is an example of a negative control.

(iii) *Genotype specific controls.* Examples of genotype specific positive controls are methyl methanesulfonate for *polA* strains and mitomycin C for *rec* strains.

(iv) *Positive controls to ensure the efficacy of the activation system.* The positive control reference substance for tests including a metabolic activation system should be selected on the basis of the type of activation system used in the test.

(v) *Other positive controls.* Other positive control reference substances may be used.

(2) *Test chemicals*—(i) *Vehicle.* Test chemicals and positive and negative control reference substances should be dissolved in an appropriate vehicle and then further diluted in vehicle for use in the assay.

(ii) *Exposure concentrations.* The test should initially be performed over a broad range of concentrations. Among the criteria to be taken into consideration for determining the upper limits of test chemical concentration are cytotoxicity and solubility. Cytotoxicity of the test chemical may be altered in the presence of metabolic activation systems. For freely soluble nontoxic chemicals, the upper test chemical concentration should be determined on a case by case basis. Because results are expressed as diameters of zones of growth inhibition in the diffusion test, it is most important that the amounts of chemical on the disc (or in the wells) are exact replicates. When appropriate, a positive response should be confirmed by testing over a narrow range of concentrations.

(e) *Test performance*—(1) *Diffusion assay*—(i) *Disc diffusion assays.* Disc diffusion assays. may be performed in two ways:

(A) A single strain of bacteria may be added to an agar overlay or spread on the surface of the agar and the test chemical placed on a filter disc on the surface of the agar or;

(B) DNA repair proficient and DNA repair deficient bacteria may be streaked in a line on the surface of the agar of the same plate and a disc saturated with test chemical placed on the surface of the agar in contact with the streaks.

(ii) *Well diffusion assays.* In well diffusion assays, bacteria may be either added to the agar overlay or spread onto the surface of the agar. A solution of the test chemical is then placed into a well in the agar.

(2) *Suspension assays.* (i) A bacterial suspension may be exposed to the test chemical and the number of surviving bacteria determined (as colony-forming units) either as a function of time of treatment or as a function of the concentration of test agent.

(ii) Nonturbid suspensions of bacteria may be exposed to serial dilutions of the test agent and a minimal inhibitory concentration for each strain determined, as evidenced by the presence or absence of visible growth after a period of incubation.

(iii) Paired bacterial suspensions (usually with some initial turbidity) may be treated with a single dose of the chemical. Positive results are indicated by a differential inhibition in the rate of increase of turbidity of the paired cultures.

(3) *Number of cultures.* When using a plate diffusion procedure, at least two independent plates should be used at each dilution. In liquid suspension assays, at least two independent specimens for determination of the number of viable cells should be plated.

(4) *Incubation conditions.* All plates in a given test should be incubated for the same time period. This incubation period should be for 18 to 24 hrs at 37 °C.

(f) *Data and report*—(1) *Treatment of results*—(i) *Diffusion assays.* Results should be expressed in diameters of zones of growth inhibition in millimeters or as areas derived therefrom as $mm^2$. Dose-response data, if available, should be presented using the same units.

(ii) *Liquid suspension assays.* (A) Survival data can be presented as dose responses, preferably as percentage of survivors or fractional survival of each strain or as a relative survival (ratio) of the two strains.

(B) Results can also be expressed as the concentrations required to effect a predetermined survival rate (e.g., $D_{37}$, the dose permitting 37 percent survival). These data are derived from the survival curve. The concentration should be expressed as weight per volume, as moles, or as molarity.

(C) Similarly, results can be expressed as minimal inhibitory concentration or as minimal lethal dose. The former is determined by the absence of visible growth in liquid medium and the latter is determined by plating dilutions onto semisolid media.

(iii) In all tests, concentrations must be given as the final concentrations during the treatment. Raw data, prior to transformation, should be provided. These should include actual quantities measured, e.g., neat numbers. For measurement of diffusion, the diameters of the discs and/or well should be indicated and the measurements should indicate whether the diameter of the discs and/or well was subtracted. Moreover, mention should be made as to whether the test chemical gave a sharp, diffuse, or double-zone of growth inhibition. If it is the latter, the investigator should indicate whether the inner or the outer zone was measured.

(iv) Viability data should be given as the actual plate counts with an indication of the dilution used and the volume plated or as derived titers (cells per ml). Transformed data alone in the absence of experimental data are not acceptable (i.e, ratios, differences, survival fraction).

(2) *Statistical evaluation.* Data should be evaluated by appropriate statistical methods.

(3) *Interpretation of results.* (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related preferential inhibition or killing of the repair deficient strain. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test points.

(ii) A test substance which does not produce either a statistically significant dose-related preferential inhibition or killing of the repair deficient strain or a statistically significant and reproducible positive response at any one of the test points is considered not to interact with the genetic material of the organisms used in assay.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(4) *Test evaluation.* DNA damage tests in bacteria do not measure DNA repair *per se* nor do they measure mutations. They measure DNA damage which is expressed as cell killing or growth inhibition. A positive result in a DNA damage test in the absence of a positive result in another system is difficult to evaluate in the absence of a better data base.

(5) *Test report.* In addition to the reporting recommendations as specified under 40 CFR Part 792, Subpart J the following specific information should be reported:

(i) Bacterial strains used.

(ii) Phase of bacterial cell growth at time of use in the assay.

(iii) Media composition.

(iv) Details of both the protocol used to prepare the metabolic activation system and its use in the assay.

(v) Treatment protocol, including doses used and rationale for dose selection, positive and negative controls.

(vi) Method used for determination of degree of cell kill.

(vii) Dose-response relationship, if applicable.

(g) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Ames, B.N., McCann, J., Yamasaki, E. "Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test," *Mutation Research,* 31:347-364 (1975).

(2) Kada, T., Sadie, Y., Tutikawa, K. "In vitro and host-mediated "rec-assay" procedures for screening chemical mutagens; and phloxine, a mutagenic red dye detected," *Mutation Research,* 16:165-174 (1972).

(3) Leifer, Z., Kada, T., Mandel, M., Zeiger, E., Stafford, R., Rosenkranz, H.S. "An evaluation of bacterial DNA repair tests for predicting genotoxicity and carcinogenicity: A report of the U.S. EPA's Gene-Tox Program," *Mutation Research,* 87:211-297 (1981).

(4) Slater, E.E., Anderson, M.D., Rosenkranz, H.S. "Rapid detection of mutagens and carcinogens." *Cancer Research,* 31:970-973 (1971).

### § 798.5550 Unscheduled DNA synthesis in mammalian cells in culture.

(a) *Purpose.* Unscheduled DNA synthesis (UDS) in mammalian cells in culture measures the repair of DNA damage induced by a variety of agents including chemicals, radiation and viruses. UDS may be measured in both in vitro and *in vivo* systems.

(b) *Definition.* In this guideline, unscheduled DNA synthesis in mammalian cells in culture is defined as the incorporation of tritium labelled thymidine ($^3$H-TdR) into the DNA of cells which are not in the S phase of the cell cycle.

(c) *Reference substances.* These may include, but need not be limited to, 7,12-dimethylbenzanthracene, 2-acetylaminofluorene, 4-nitroquinoline oxide or N-dimethyl-nitrosamine.

(d) *Test method*—(1) *Principle.* Mammalian cells in culture, either primary cultures of rodent hepatocytes or established cell lines, are exposed to the test agent. Established cell lines are treated both with and without metabolic activation. UDS is measured by the uptake of $^3$H-TdR into the DNA of non-S phase cells. Uptake may be determined by autoradiography or by liquid scintillation counting (LSC) of DNA from treated cells.

(2) *Description*—(i) *Autoradiography.* For autoradiography, coverslip cultures of cells are exposed to test chemical in medium containing $^3$H-TdR. At the end of the treatment period, cells are fixed, dipped in autoradiographic emulsion, and exposed at 4 °C. At the end of the exposure period, cells are stained and labeled nuclei are counted either manually or with an electronic counter. Established cell lines should be treated both with and without metabolic activation.

(ii) *LSC determinations.* For LSC determinations of UDS, confluent cultures of cells are treated with test chemical both with and without metabolic activation. At the end of the exposure period, DNA is extracted from the treated cells. Total DNA content is determined and extent of $^3$H-TdR incorporation is determined by scintillation counting.

(3) *Cells*—(i) *Type of cells used in the assay.* (A) A variety of cell lines or primary cell cultures, including human cells, may be used in the assay.

(B) Established cell lines should be checked for *Mycoplasma* contamination and may be periodically checked for karyotype stability.

(ii) *Cell growth and maintenance.* Appropriate culture media and incubation conditions (culture vessels $CO_2$ concentration), temperature and humidity should be used.

(4) *Metabolic activation.* (i) A metabolic activation system is not used with primary cultures of rodent hepatocytes.

(ii) Established cell lines should be exposed to test substance both in the presence and absence of an appropriate metabolic activation system.

(5) *Control groups.* Concurrent positive and negative (untreated and/or vehicle) controls both with and without metabolic activation as appropriate should be included in each experiment.

(6) *Test chemicals*—(i) *Vehicle.* Test chemicals and positive control reference substances may be prepared in culture media or dissolved or suspended in appropriate vehicles prior to treatment of the cells. Final concentration of the vehicle should not interfere with cell viability or growth rate.

(ii) *Exposure concentrations.* Multiple concentrations of test substance, based upon cytotoxicity and over a range adequate to define the response, should be used. For cytotoxic chemicals, the first dose to elicit a cytotoxic response in a preliminary assay should be the highest dose tested. Relatively insoluble compounds should be tested up to the limits of solubility. For freely soluble nontoxic chemicals, the upper test chemical concentration should be determined on a case by case basis.

(e) *Test performance*—(1) *Primary cultures of rodent hepatocytes.* Freshly isolated rodent hepatocytes should be treated with chemical in medium containing $^3$H-TdR. At the end of the treatment period, cells should be drained of medium, rinsed, fixed, dried and attached to microscope slides. Slides should be dipped in autoradiographic emulsion, exposed at 4 °C. for an appropriate length of time, developed, stained and counted.

(2) *Established cell lines*—(i) *Autoradiographic techniques.* The techniques for treatment of established cell lines are the same as those for primary cultures of rodent hepatocytes except that cells must not enter S phase prior to treatment. Entry of cells into S phase may be blocked by several methods (e.g. by growth in medium deficient in arginine or low in serum or by treatment with chemical agents such as hydroxyurea). Tests should be done both in the presence and absence of a metabolic activation system.

(ii) *LSC measurement of UDS.* Prior to treatment with test agent, entry of cells into S phase should be blocked as described in paragraph (e)(2)(i) of this section. Cells should be exposed to the test chemical in medium containing $^3$H-TdR. At the end of the incubation period, DNA should be extracted from the cells by hydrolysis with perchloroacetic acid or by other acceptable methods. One aliquot of DNA is used to determine total DNA content; a second aliquot is used to measure the extent of $^3$H-TdR incorporation.

(3) *Acceptable background frequencies*—(i) *Autoradiographic determinations.* Net incorporation of $^3$HTdR into the nucleus of solvent treated control cultures should be less than 1.

(ii) *LSC determinations.* Historical background incorporation rates of $^3$H-TdR into untreated established cell lines should be established for each laboratory.

(4) *Number of cells counted.* A minimum of 50 cells per culture should be counted for autoradiographic UDS determinations. Slides should be coded before being counted. Several widely separated random fields should be counted on each slide. Cytoplasm adjacent to the nuclear areas should be counted to determine spontaneous background.

(5) *Number of cultures.* Six independent cultures at each concentration and control should be used in LSC UDS determinations.

(f) *Data and report*—(1) *Treatment of results*—(i) *Autoradiographic determinations.* For autoradiographic determinations, once untransformed data are recorded, background counts should be subtracted to give the correct nuclear grain count. Values should be reported as net grains per nucleus. Mean, median and mode may be used to describe the distribution of net grains per nucleus.

(ii) *LSC determinations.* For LSC determinations, $^3$H-TdR incorporation should be reported as dpm/μg DNA. Average dpm/μg DNA with standard deviation or standard error of the mean may be used to describe distribution of incorporation in these studies.

(2) *Statistical evaluation.* Data should be evaluated by appropriate statistical methods.

(3) *Interpretation of results.* (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the incorporation of $^3$H-TdR into treated cells. Another criterion may be based upon detection of a reproducible and statistically significant positive response for a least one of the test points.

(ii) A test substance which does not produce either a statistically significant dose-related increase in the incorporation of $^3$H-TdR into treated cells or a statistically significant and reproducible positive response at any one of the test points is considered not to induce UDS in the test system.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(4) *Test evaluation.* (i) Positive results in the UDS assay indicate that under the test conditions the test substance may induce DNA damage in cultured mammalian somatic cells.

(ii) Negative results indicate that under the test conditions the test substance does not induce DNA damage in cultured mammalian somatic cells.

(5) *Test report.* In addition to the reporting recommendations as specified under 40 CFR Part 792, Subpart J the following specific information should be reported:

(i) Cells used, density and passage number at time of treatment, number of cell cultures.

(ii) Methods used for maintenance of cell cultures including medium, temperature and $CO_2$ concentration.

(iii) Test chemical vehicle, concentration and rationale for selection of concentrations used in the assay.

(iv) Details of both the protocol used preparation of the metabolic activation system and its use in the assay.

(v) Treatment protocol.

(vi) Positive and negative controls.

(vii) Protocol used for autoradiography.

(viii) Details of the method used to block entry of cells into S phase.

(ix) Details of the methods used for DNA extraction and determination of total DNA content in LSC determinations.

(x) Historical background incorporation rates of ³H-TdR in untreated cell lines.

(xi) Dose-response relationship, if applicable.

(g) *References.* For additional background information on this test guideline, the following references should be consulted:

(1) Ames, B.N., McCann, J., Yamasaki, E. "Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test," *Mutation Research*, 31:347-364 (1975).

(2) Rasmussen, R.E., Painter, R.B. "Radiation-stimulated DNA synthesis in cultured mammalian cells," *Journal of Cell Biology*, 29:11-19 (1966).

(3) Stich, H.F., San, P.P.S., Lam, K.J., Koropatnick, D.J., Lo, L.W., Laishes, B.A. "DNA fragmentation and DNA repair as an in vitro and in vivo assay for chemical procarcinogens, carcinogens and carcinogenic nitrosation products," *Screening tests in chemical carcinogenesis*. Eds. Bartsch, H., Tomatis, L., (Lyon: IARC Scientific Publications, No. 12, 1976) pp. 617-636.

(4) Williams, G.M. "Carcinogen-induced DNA repair in primary rat liver cell cultures: a possible screen for chemical carcinogens," *Cancer Letters*, 1:231-236 (1976).

(5) Williams, G.M. "Detection of chemical carcinogens by unscheduled DNA synthesis in rat liver primary cell cultures," *Cancer Research*, 37:1845-1851 (1977).

## § 798.5575 Mitotic gene conversion in Saccharomyces cerevisiae.

(a) *Purpose.* The mitotic gene conversion assay in the yeast, *Saccharomyces cerevisiae*, measures the conversion of differentially inactive alleles to
wild-type alleles by mutagenic ager
Heteroallelic diploid yeast stra
carry two different inactive alleles
the same gene locus. The presence
these alleles causes a nutritional
quirement, e.g., these heteroallelic
ploids grow only in medium sup
mented with a specific nutrient s
as tryptophan. When gene convers
occurs, a fully active wild-type phe
type is produced from these inac
alleles through intragenic recomb
tion. These wild-type colonies grow
a medium lacking the specific nu
tional requirement (selective mediu

(b) *Definitions.* (1) Mitotic gene c
version is detected by the change
inactive alleles of the same gene
wild-type alleles through intrage
recombination in mitotic cells.

(2) Heteroallelic diploids are dip
strains of yeast carrying two differe
inactive alleles of the same gene lo
causing a nutritional requirement.

(c) *Reference substances.* These r
include but need not be limited to,
drazine sulfate or 2-acetylaminoflu
ene.

(d) *Test method*—(1) *Principle.* T
method is based on the fact that
teroallelic diploid yeast strains ca
two inactive alleles of the same g
locus making them dependent o
specific nutritional requirement (
tryptophan) for their survival. Tr
ment of such strains with mutag
agents can cause conversion of th
alleles back to the wild-type condi
which allows growth on a med
lacking the required nutrient (se
tive medium).

(2) *Description.* Heteroallelic dip
strains such as D7, requiring a spe
nutrient in the medium, are trea
with test chemical with and with
metabolic activation and plated o
selective medium lacking the requ
nutrient. The wild-type colonies
grow on the selective medium
result of gene conversion are score

(3) *Strain selection*—(i) *Designat*
At the present time, *S. cerevi*
strain D7 is recommended for us
this assay. The use of other str
may also be appropriate.

(ii) *Preparation and storage.* S
culture preparation and stor
growth requirements, method of st
identification and demonstration

appropriate phenotypic requirements should be performed using good microbiological techniques and should be documented.

(iii) *Media.* YEP glucose medium enriched with the appropriate growth factors may be used for cell growth and maintenance. Other media may also be appropriate.

(4) *Selection of cultures.* Cells should be grown with aeration in liquid medium enriched with growth factors to early stationary phase. Cells should then be seeded on selective medium to determine the rate of spontaneous conversion. Cultures with a high rate of spontaneous conversion should be discarded.

(5) *Metabolic activation.* Cells should be exposed to test chemical both in the presence and absence of an appropriate metabolic activation system.

(6) *Control groups.* Concurrent positive and negative (untreated and/or vehicle) controls both with and without metabolic activation should be included in each experiment.

(7) *Test chemicals*—(i) *Vehicle.* Test chemicals and positive control reference substances should be dissolved in an appropriate vehicle and then further diluted in vehicle for use in the assay. Dimethylsulfoxide should be avoided as a vehicle.

(ii) *Exposure concentrations.* (A) The test should initially be performed over a broad range of concentrations. Among the criteria to be taken into consideration for determining the upper limits of test chemical concentration are cytotoxicity and solubility. Cytotoxicity of the test chemical may be altered in the presence of metabolic activation systems. For cytotoxic chemicals, the highest dose tested should not reduce survival to less than 10 percent of that seen in the untreated control cultures. Relatively insoluble chemicals should be tested up to the limits of solubility. For freely soluble nontoxic chemicals, the upper test chemical concentration should be determined on a case by case basis.

(B) When appropriate, a positive response should be confirmed by using a narrow range of concentrations.

(e) *Test performance*—(1) *Treatment.* Cultures should be treated in liquid suspension. Resting cells should be treated in buffer; growing cells should be treated in a synthetic medium. Cultures with low spontaneous convertant frequencies should be centrifuged, washed and resuspended in liquid at the appropriate density. Cells should be exposed to test chemical both in the presence and absence of a metabolic activation system. Independent tubes should be treated for each concentration. At the end of the treatment period, cells should be centrifuged, washed and resuspended in distilled water prior to plating on selective medium for convertant selection and on complete medium to determine survival. At the end of the incubation period, plates should be scored for survival and the presence of convertant colonies.

(2) *Number of cultures.* At least six individual plates per treatment concentration and control should be used.

(3) *Incubation conditions.* All plates in a given experiment should be incubated for the same time period. This incubation period may be from 4 to 6 days at 28 °C.

(f) *Data and report*—(1) *Treatment of results.* Individual plate counts for test substance and control should be presented for both convertants and survivors. The mean number of colonies per plate and standard deviation should also be presented. Data should be presented in tabular form indicating numbers of viable and convertant colonies scored, survival frequency and convertant frequencies for each treatment and control culture. Conversion frequencies should be expressed as number of convertants per number of survivors. Sufficient detail should be provided for verification of survival and convertant frequencies.

(2) *Statistical evaluation.* Data should be evaluated by appropriate statistical methods.

(3) *Interpretation of results.* (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of gene convertants. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test points.

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of gene conversions or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(4) *Test evaluation.* (i) Positive results in this assay indicate that under the test conditions the test chemical causes mitotic gene conversion in the yeast *S. cerevisiae.*

(ii) Negative results indicate that under the test conditions the test chemical does not cause mitotic gene conversion in *S. cerevisiae.*

(5) *Test report.* In addition to the reporting recommendations as specified under 40 CFR Part 792, Subpart J, the following specific information should be reported:

(i) Strain of organism used in the assay.

(ii) Test chemical vehicle, doses used and rationale for dosage selection.

(iii) Method used to select cultures.

(iv) Treatment protocol including cell density at treatment and length of exposure to test substance.

(v) Details of both the protocol used to prepare the metabolic activation system and its use in the assay.

(vi) Incubation times and temperatures.

(vii) Dose-response relationship, if applicable.

(g) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Ames, B.N., McCann, J., Yamasaki, E. "Methods for detecting carcinogens and mutagens with the *Salmonella/*mammalian-microsome mutagenicity test," *Mutation Research,* 31:347-364 (1975).

(2) Callen, D.F., Philpot, R.M. "Cytochrome P-450 and the activation of promutagens in *Saccharomyces cerevisiae,*" *Mutation Research,* 45:309-324 (1975).

(3) Zimmermann, F.K. "Procedures used in the induction of mitotic recombination and mutation in the yeast *Saccharomyces cerevisiae.*" *Handbook*
*of mutagenicity test procedures.* E
Kilby, B.J., Legator, M., Nicols,
Ramel, C, (Amsterdam: Elsevi
North Holland Biomedical Pr
(1979), pp. 119-134.

(4) Zimmermann, F.K., Kern,
Rosenberger, H. "A yeast strain for
multaneous detection of induced
totic crossing over, mitotic gene c
version and reverse mutation," *Mu*
*tion Research,* 28:381-388 (1975).

### § 798.5900 In vitro sister chromatid change assay.

(a) *Purpose.* The sister chroma
exchange (SCE) assay detects the a
ity of a chemical to enhance the
change of DNA between two si
chromatids of a duplicating chro
some. The test may be performed
vitro, using, for example, rodent
human cells, or in vivo using m
mals, for example, rodents such
mice, rats and hamsters.

(b) *Definition.* Sister chromatid
changes represent reciprocal in
changes of the two chromatid a
within a single chromosome. These
changes are visualized during
metaphase portion of the cell c
and presumably require enzymatic
cision, translocation and ligation o
least two DNA helices.

(c) *Reference substances.* Not ap
cable.

(d) *Test method*—(1) *Principle.*
lowing exposure of cell cultures to
chemicals, they are allowed to r
cate in the presence of bromodeox
idine (BrdU), followed by treatm
with colchicine or colcemid to ar
cells in a metaphase-like stage of m
sis (c-metaphase). Cells are then
vested and chromosome preparat
made. Preparations are stained
metaphase cells analyzed for SCEs.

(2) *Description. In vitro* SCE as
may employ monolayer or suspen
cultures of established cell lines,
strains or primary cell cultures.
cultures are exposed to test chem
and are allowed to replicate in
presence of BrdU. Prior to har
cells are treated with a spindle in
tor (e.g. Colchicine or Colcemid®
accumulate cells in c-metaphase. C
mosome preparations from cells
made, stained and analyzed for S

(3) *Cells*—(i) *Type of cells used in the assay.* There are a variety of cell lines or primary cell cultures, including human cells, which may be used in the assay. Established cell lines and strains should be checked for *Mycoplasma* contamination and may be periodically checked for karyotype stability.

(ii) *Cell growth and maintenance.* Appropriate culture media and incubation conditions (culture vessels, temperature, and humidity $CO_2$) should be used.

(4) *Metabolic activation.* Cells should be exposed to test chemical both in the presence and absence of an appropriate metabolic activation system.

(5) *Control groups—Concurrent controls.* Positive and negative (untreated and/or vehicle) controls, with and without metabolic activation, should be included in each experiment.

(6) *Test chemicals*—(i) *Vehicle.* Test substances may be prepared in culture media or dissolved or suspended in appropriate vehicles prior to treatment of the cells. Final concentration of the vehicle should not reduce cell viability or growth rate.

(ii) *Exposure concentrations.* Multiple concentrations of the test substance over a range adequate to define the response should be tested. Among the criteria to be taken into consideration for determining the upper limits of test chemical concentration are cytotoxicity and solubility. Cytotoxicity of the test substance may be altered in the presence of metabolic activation systems. Cytotoxicity may be evidenced by a large (e.g., 75 percent) decrease in the number of cells that have divided twice in the presence of BrdU. Relatively insoluble substances should be tested up to the limit of solubility. For freely soluble nontoxic chemicals, the upper test chemical concentration should be determined on a case by case basis. When appropriate, a positive response should be confirmed by using a narrow range of test concentrations.

(e) *Test performance*—(1) *Established cell lines and strains.* (i) Prior to use in the assay, cells should be generated from stock cultures, seeded in culture vessels at the appropriate density and incubated at 37 °C.

(ii) Cell lines and strains should be treated with test chemical both with and without metabolic activation when they are in the exponential stage of growth. At the end of the exposure period, cells should be washed and incubated for two replication cycles in medium containing BrdU. After BrdU is added, the cultures should be handled in darkness, under "safe" (e.g., darkroom) lights, or in dim light from incandescent lamps to minimize photolysis of BrdU containing DNA. At the end of the BrdU incubation period, cells should be fixed and stained for SCE determination. Cultures should be treated with a spindle inhibitor (e.g., colchicine or Colcemid®) 2 hr prior to harvesting.

(2) *Human lymphocyte cultures.* (i) For preparation of human lymphocyte cell cultures, heparinized or acid-citrate-dextrose treated whole blood should be added to culture medium containing a mitogen, e.g., phytohemagglutinin (PHA) and incubated at 37 °C. White cells sedimented by gravity (buffy coat) or lymphocytes which have been purified on a density gradient such as Ficoll-Hypaque may also be utilized.

(ii) Cells should be exposed to the test chemical during at last two time intervals, e.g. $G_0$ and S. Exposure during the $G_0$ phase of the cell cycle should be accomplished by adding the test substance prior to addition of mitogen. Exposure during or after the first S phase may be accomplished by exposing cells 24-30 hrs after mitosis, under "safe" (e.g. darkroom) lights, or in dim light from incandescent lamps to minimize photolysis of BrdU containing DNA. At the end of the BrdU incubation period, cells should be fixed and stained for SCE determination. Cultures should be treated with a spindle inhibitor (e.g. colchicine or Colcemid®) 2 hr prior to harvesting.

(3) *Human lymphocyte cultures.* (i) For preparation of human lymphocyte cell cultures, heparinized or acid-citrate-dextrose treated whole blood should be added to culture medium containing a mitogen, e.g., phytohemagglutinin (PHA) and incubated at 37 °C. White cells sedimented by gravi-

ty (buffy coat) or lymphocytes which have been purified on a density gradient such as Ficoll-Hypaque may also be utilized.

(ii) Cells should be exposed to the test chemical during at least two time intervals, e.g., $G_0$ and S. Exposure during the $G_0$ phase of the cell cycle should be accomplished by adding the test substance prior to addition of mitogen. Exposure during or after the first S phase may be accomplished by exposing cells 24–30 hrs after mitogen stimulation. After exposure, cells should be washed and then cultured in the absence of the chemical.

(4) *Culture harvest time.* A single harvest time, one that yields an optimal percentage of second division metaphases, is recommended. If there is reason to suspect that this is not a representative sampling time (which may occur for short-lived, cycle specific chemicals), then additional harvest times should be selected.

(5) *Staining method.* Staining of slides to reveal SCEs can be performed according to any of several protocols. However, the fluorescence plus Giemsa method is recommended.

(6) *Number of cultures.* At least two independent cultures should be used for each experimental point.

(7) *Analysis.* Slides should be coded before analysis. The number of cells to be analyzed should be based upon the spontaneous control frequency and defined sensitivity and the power of the test chosen before analysis. In human lymphocytes, only cells containing 46 centromeres should be analyzed. In established cell lines and strains, only metaphases containing ±2 centromeres of the modal number should be analyzed. Uniform criteria for scoring SCEs should be used.

(f) *Data and report*—(1) *Treatment of results.* Data should be presented in tabular form, providing scores for both the number of SCEs for each metaphase and the number of SCEs per chromosome for each metaphase.

(2) *Statistical evaluation.* Data should be evaluated by appropriate statistical methods.

(3) *Interpretation of results.* (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-relat-
ed increase in the number of s
chromatid exchanges. Another c
rion may be based upon detection
reproducible and statistically sig
cant positive response for at least
of the test substance concentration

(ii) A test substance which prod
neither a statistically significant d
related increase in the numbe
sister chromatid exchanges nor a
tistically significant and reprodu
positive response at any one of
test points is considered not to in
rearrangements of segments of
in this system.

(iii) Both biological and statis
significance should be considered
gether in the evaluation.

(4) *Test evaluation.* (i) Positive
sults in the in vitro SCE assay ind
that under the test conditions the
substance induces reciprocal chr
tid interchanges in cultured mam
an somatic cells.

(ii) Negative results indicate
under the test conditions the test
stance does not induce reciprocal c
matid interchanges in cultured m
malian somatic cells.

(5) *Test report.* In addition to th
porting recommendations as spec
under 40 CFR Part 792, Subpart J
following specific information sh
be reported:

(i) Cells used, density at tim
treatment, number of cell cultures

(ii) Methods used for maintenan
cell cultures including medium,
perature and $CO_2$ concentration.

(iii) Test chemical vehicle, con
tration and rationale for the selec
of the concentrations of test chem
used in the assay, duration of t
ment.

(iv) Details of both the protocol
preparation of the metabolic ac
tion system and its use in the assa

(v) Growth period in BrdU; ide
of spindle inhibitor, its concentra
and duration of treatment.

(vi) Time of cell harvest.

(vii) Positive and negative contro

(viii) Method used to prepare s
for SCE determination.

(ix) Criteria for scoring SCEs.

(x) Details of the protocol used
growth and treatment of human
if used in the assay.

(xi) Dose-response relationship, if applicable.

(g) *References.* For additional background information on this test guideline the following references shoud be consulted:

(1) Latt, S.A., Allen, J., Bloom, S.E., Carrano, A., Falke, E., Kram, D., Schneider, E., Schreck, R., Tice, R., Whitfield, B., Wolff, S. "Sister chromatid exchanges: a report of the U.S. EPA's Gene-Tox Program" *Mutation Research*, 87:17-62 (1981).

(2) [Reserved]

### § 798.5915 In vivo sister chromatid exchange assay.

(a) *Purpose.* The sister chromatid exchange (SCE) assay detects the ability of a chemical to enhance the exchange of DNA between two sister chromatids of a duplicating chromosome. The test may be perfomed in vitro using cultured mammalian cells or in vivo using nonmammalian or mammalian tissues. The most commonly used assays employ bone marrow or lymphocytes from mammalian species such as mice, rats or hamsters. Human lymphocytes may also be used.

(b) *Definition.* Sister chromatid exchanges represent reciprocal interchanges of the two chromatid arms within a single chromosome. These exchanges are visualized during the metaphase portion of the cell cycle and presumably require enzymatic incision, translocation and ligation of at least two DNA helices.

(c) *Reference substances.* Not applicable.

(d) *Test method*—(1) *Principle.* Animals are exposed to test substance by appropriate routes followed by administration of bromodeoxyuridine (BrdU). A spindle inhibitor (e.g., colchicine or Colecimid®) is administered prior to sacrifice. After sacrifice, tissue is obtained and metaphase preparations made, stained and scored for SCE.

(2) *Description.* The method described here employs bone marrow of laboratory rodents exposed to test chemicals. After treatment with test chemical, animals are futher treated with BrdU and, prior to sacrifice, with a spindle inhibitor (e.g., colchicine or Colcemid®) to arrest cells in c-metaphase. After sacrifice, chromosome preparations from bone marrow cells are made, stained and scored for SCE.

(3) *Animal selection*—(i) *Species and strain.* Any appropriate mammalian species may be used. Examples of commonly used rodent species include mice, rats, and hamsters.

(ii) *Age.* Healthy, young adult animals should be used.

(iii) *Number and sex.* At least five female and five male animals per experimental and control group should be used. The use of a single sex or different number of animals should be justified.

(iv) *Assignment to groups.* Animals should be randomized and assigned to treatment and control groups.

(4) *Control groups*—(i) *Concurrent controls.* Current positive and negative (vehicle) controls should be included in the assay.

(ii) *Positive controls.* A compound know to produce SCE *in vivo* should be employed as the positive control.

(5) *Test chemicals*—(i) *Vehicle.* When possible, test chemicals should be dissolved in isotonic saline or distilled water. Water insoluble chemicals may be dissolved or suspended in appropriate vehicles. The vehicle used should neither interfere with the test compound nor produce toxic effects. Fresh preparations of the test compound should be employed.

(ii) *Dose levels.* For an initial assessment, one dose of the test substance may be used, the dose being the maximum tolerated dose or that producing some indication of toxicity as evidenced by animal morbidity (including death) or target cell toxicity. The LD$_{50}$ is a suitable guide. Additional dose levels may be used. For determination of dose-response, at least three dose levels should be used.

(iii) *Route of administration.* The usual routes of administration are IP or oral. Other routes may be appropriate.

(iv) *Treatment schedule.* In general, test substances should be administered only once. However, based upon toxicological information a repeated treatment schedule may be employed.

(e) *Test performance*—(1) *Treatment.* Animals should be treated with test chemical followed by administration of BrdU. BrdU may be administered by multiple IP injections, by continuous tail vein infusion or by subcutaneous implantation of tablets. Animals should be treated with a spindle inhibitor (e.g. colchicine or Colcemid®) 2 hr prior to sacrifice. After sacrifice, bone marrow should be extracted and slides made and prepared for SCE evaluation.

(2) *Staining method.* Staining of slides to reveal SCEs can be performed according to any of several protocols. However, the fluorescence plus Giemsa method is recommended.

(3) *Number of cells scored.* The number of cells to be analyzed per animal should be based upon the number of animals used, the negative control frequency, the predetermined sensitivity and the power chosen for the test. Slides should be coded before microscopic analysis.

(f) *Data and report*—(1) *Treatment of results.* Data should be presented in tabular form, providing scores for both the number of SCE for each metaphase and the number of SCE per chromosome for each metaphase. Differences among animals within each group should be considered before making comparisons between treated and control groups.

(2) *Statistical evaluation.* Data should be evaluated by appropriate statistical methods.

(3) *Interpretation of results.* (i) There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of SCE. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test points.

(ii) A test substance which does not produce either a statistically significant dose-related increase in the number of SCE or a statistically significant and reproducible positive response at any one of the test points is considered not to induce rearrangements of DNA segments in this system.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(4) *Test evaluation.* (i) Positive
sults in the *in vivo* SCE assay indic
that under the test conditions the t
substance induces reciprocal int
changes in the bone marrow of t
test species.

(ii) Negative results indicate th
under the test conditions the test s
stance does not induce recipro
interchanges in the bone marrow
the test species.

(5) *Test report.* In addition to the
porting recommendations as specif
under 40 CFR Part 792, Subpart J, t
following specific information sho
be reported:

(i) Species, strain, age, weig
number and sex of animals in e
treatment and control group.

(ii) Test chemical vehicle, dose le
used, rationale for dose selection, t
icity data, negative and positive c
trols.

(iii) Route and schedule of admi
tration of both test chemical
BrdU.

(iv) Identity of spindle inhibitor,
concentration and duration of tr
ment.

(v) Time of sacrifice after admi
tration of BrdU.

(vi) Details of the protocol used
slide preparation.

(vii) Criteria for scoring SCE.

(viii) Dose-response relationship
applicable.

(g) *References.* For additional b
ground information on this test gu
line the following references shoul
consulted:

(1) Allen, J.W., Shuler, C.F., L
S.A. "Bromodeoxyuridine tablet m
odology for in vivo studies of D
synthesis," *Somatic Cell Gene*
4:393-405 (1978).

(2) Allen, J.W., Shuler, C.F., Men
R.W., Latt, S.A. "Simplified techn
for in vivo analysis of sister chrom
exchanges using 5-bromodeoxyuri
tablets" *Cytogenetics Cell Gene*
18:231-237 (1977).

(3) Latt, S.A., Allen, J., Bloom,
Carrano, A., Falke, E., Kram,
Schneider, E., Schreck, R., Tice,
Whitfield, B., Wolff, S. "Sister c
matid exchanges: A report of the
EPA Gene-Tox Program," *Muta*
*Research*, 87:17-62 (1981).

## § 798.5955 Heritable translocation test in drosophila melanogaster.

(a) *Purpose.* The heritable translocation test in *Drosophila* measures the induction of chromosomal translocations in germ cells of insects. Stocks carrying genetic markers on two or more chromosomes are used to follow the assortment of chromosomes in meiosis. The $F_1$ male progeny of treated parents are individually mated to females and the $F_2$ progeny phenotypes are scored. The observed spectrum of phenotypes is used to determine the presence or absence of a translocation. This is usually indicated by a lack of independent assortment of genes on different chromosomes.

(b) *Definitions*—(1) Chromosome mutations are chromosomal changes resulting from breakage and reunion of chromosomes. Chromosomal mutations are also produced through nondisjunction of chromosomes during cell division.

(2) Reciprocal translocations are chromosomal translocations resulting from reciprocal exchanges between two or more chromosomes.

(3) Heritable translocations are reciprocal translocations transmitted from parent to the succeeding progeny.

(c) *Reference substances.* These may include, but need not be limited to, ethyl methanesulfonate or N-dimethyl-nitrosamine.

(d) *Test method*—(1) *Principle.* The method is based on the principle that balanced reciprocal chromosomal translocations can be induced by chemicals in the germ cells of treated flies and that these translocations are detected in the $F_2$ progeny using genetic markers (mutations). Different mutations may be used as genetic markers and two or more of the four chromosomes may be genetically marked for inclusion in this test.

(2) *Description.* Wild-type males are treated with chemical and bred with females of known genetic markers. The $F_1$ males are collected and individually bred with virgin females of the female parental stock. The resulting $F_2$ progeny are scored. Putative translocation carriers are confirmed with an $F_3$ cross.

(i) *Illustrative example.* The following example serves to illustrate the method. Males carrying genes for red eye color on chromosomes II and III are bred with females of white eye color carrying alleles for brown (bw) on the second chromosome and scarlet (st) and pink (pp) on the third chromosome. The $F_1$ male progeny are bred with virgin females of the female parental stock and the resulting $F_2$ progeny are examined for eye color phenotypes. If there is no translocation in the $F_1$ male, then the resulting $F_2$ progeny will have four eye color phenotypes: red, white, orange, and brown. If the $F_1$ male carries a translocation between chromosomes II and III, only red and white eye phenotypes are obtained in the $F_2$ generation. This happens because the $F_1$ translocation heterozygote produces two balanced (carrying either the parental or the translocated configuration of markers) and two unbalanced gametes. The unbalanced gametes (carrying one normal and one translocated chromosome) are unable to develop into normal individuals in the $F_2$ generation.

(ii) [Reserved]

(3) *Drosophila stocks.* Wild-type males and females of the genotype bw;st;pp (white eyes) may be used in the heritable translocation test. Other appropriately marked *Drosophila* stocks may also be used.

(4) *Control groups.* (i) Concurrent positive and negative (vehicle) controls should be included in each experiment.

(ii) Negative (vehicle) controls should be included. The size of the negative (vehicle) control group should be determined by the availability of appropriate laboratory historical control data.

(iii) If the historical control data are of sufficient numbers, concurrent controls may not be necessary.

(5) *Test chemicals*—(i) *Vehicle.* Test chemicals should be dissolved in water. Compounds which are insoluble in water may be dissolved or suspended in appropriate vehicles (e.g., a mixture of ethanol and Tween-60 or 80), and then diluted in water or saline prior to administration. Dimethylsulfoxide should be avoided as a vehicle.

(ii) *Dose levels.* For the initial assessment of mutagenicity, it may be sufficient to test a single dose of the test substance. This dose should be the maximum tolerated dose or that which produces some indication of toxicity. If the test is being used to verify mutagenic activity, at least two additional exposure levels should be used.

(iii) *Route of administration.* Exposure may be oral, by injection or by exposure to gases or vapours. Feeding of the test compound may be done in sugar solution. When necessary, substances may be dissolved in 0.7 percent NaCl solution and injected into the thorax or abdomen.

(e) *Test performance*—(1) *P1 mating.* (i) In the primary screen of a chemical, it is enough to sample one germ cell stage, either mature sperm or spermatids (for indirect acting mutagens). Other stages may be sampled if needed, i.e., when mature germ cells give a positive result and data from earlier germ cells are needed for the purpose of risk assessment. Thus, the treated males may be mated only once for a period of 3 days to sample sperm or transferred every 2 to 3 days to cover the entire germ cell cycle.

(ii) Mass matings may be performed because the control rate for translocations in the available literature is very low (near 0) and clustered events are extremely rare. Mated females may be aged for 2 weeks in order to recover an enhanced incidence of translocation due to the storage effect. The females are then allowed to lay eggs and $F_1$ males are collected for test mating.

(2) *$F_1$ mating.* $F_1$ males should be bred with virgin females of the parental female stock. Since each $F_1$ male represents one treated gamete of the male parent, the $F_1$ males have to be mated individually to virgin females. Each $F_1$ male should be mated to three females to ensure sufficient progeny.

(3) *Scoring the $F_2$ generation.* $F_2$ cultures (each representing 1 $F_1$ male tested) should be scored for the presence or absence of phenotype variations (linkage of markers) from the expected types. The test should be designed with a predetermined sensitivity and power. The number of flies in each group should reflect these de-
fined parameters. The spontaneo
mutant frequency observed in the
propriate control group will stron
influence the number of treated ch
mosomes that must be analyzed
detect substances which show mu
tion rates close to those of the co
trols. A positive test should be co
firmed by $F_3$ mating trials.

(4) *Number of replicate experimen*
Replicate experiments are usually p
formed for each dose of the compou
tested. If a chemical is a potent ind
er of translocations, one experime
may be sufficient. Otherwise two
three replicate experiments should
done.

(f) *Data and report*—(1) *Treatm*
*of results.* Data should be tabulated
show the number of translocatio
and the number of fertile $F_1$ males
each exposure for each germ cell sta
sampled.

(2) *Statistical evaluation.* D
should be evaluated by appropri
statistical methods.

(3) *Interpretation of results.*
There are several criteria for det
mining a positive result, one of whi
is a statistically significant dose-rel
ed increase in the number of herita
translocations. Another criterion m
be based upon detection of a reprod
ible and statistically significant po
tive response for at least one of t
test points.

(ii) A test substance which does
produce either a statistically sign
cant dose-related increase in
number of heritable translocations
a statistically significant and rep
ducible positive response at any one
the test points is considered nonmu
genic in this system.

(iii) Both biological and statisti
significance should be considered
gether in the evaluation.

(4) *Test evaluation.* (i) Positive
sults in the heritable translocat
test in *Drosophila* indicate that un
the test conditions the test substa
causes chromosome damage in ge
cells of this insect.

(ii) Negative results indicate t
under the test conditions the test s
stance does not cause chromoso
damage in *D. melanogaster.*

(5) *Test report.* In addition to the
porting recommendations as specif

under 40 CFR Part 792, Subpart J, the following specific information should be reported:

(i) *Drosophila* stock used in the assay, age of insects, number of males treated, number of $F_2$ cultures established, number of replicate experiments.

(ii) Test chemical vehicle, treatment and mating schedule, exposure levels, toxicity data, dose and route of exposure.

(iii) Positive and negative (vehicle) controls.

(iv) Historical control data, if available.

(v) Number of chromosomes scored.

(vi) Criteria for scoring mutant chromosomes.

(vii) Dose-response relationship, if applicable.

(g) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Wurgler, F.E., Sobels, F.H., Vogel, E. "*Drosophila* as assay system for detecting genetic changes," *Handbook of mutagenicity test procedures.* Eds. Kilby, B.J., Legator, M., Nichols, W., Ramel, C. (Amsterdam: Elsevier/North Holland Biomedical Press, 1979) pp. 335–374.

(2) [Reserved]

## Subpart G—Neurotoxicity

### §798.6050 Functional observational battery.

(a) *Purpose.* In the assessment and evaluation of the potential human health effects of substances, it may be necessary to test for neurotoxic effects. Substances that have been observed to cause neurotoxic signs (e.g., convulsions, tremors, ataxia) in other toxicity tests, as well as those having a structural similarity to known neurotoxicants, should be evaluated for neurotoxicity. The functional observational battery is a noninvasive procedure designed to detect gross functional deficits in young adults resulting from exposure to chemicals and to better quantify neurotoxic effects detected in other studies. This battery of tests is not intended to provide a detailed evaluation of neurotoxicity. It is designed to be used in conjunction with neuropathologic evaluation and/or general toxicity testing. Additional functional tests may be necessary to assess completely the neurotoxic potential of a chemical.

(b) *Definitions.* (1) Neurotoxicity is any adverse effect on the structure or function of the central and/or peripheral nervous system related to exposure to a chemical substance.

(2) A toxic effect is an adverse change in the structure or function of an experimental animal as a result of exposure to a chemical substance.

(c) *Principle of the test method.* The material is administered by an appropriate route to laboratory rodents. The animals are observed under carefully standardized conditions with sufficient frequency to ensure the detection of behavioral and/or neurologic abnormalities, if present. Various functions that could be affected by neurotoxicants are assessed during each observation period.

(d) *Test procedures*—(1) *Animal selection*—(i) *Species and strain.* The laboratory rat or mouse is recommended. Although information will generally be lacking, whenever possible the choice of species should take into consideration such factors as the comparative metabolism of the chemical and species sensitivity to the toxic effects of the test substance, as evidenced by the results of other studies. The potential for combined studies should also be considered. Standard strains should be used.

(ii) *Age.* Young adult animals (at least 42 days old for the rat or mouse) shall be used.

(iii) *Sex.* (A) Equal numbers of animals of each sex are required for each dose level.

(B) The females shall be nulliparous and nonpregnant.

(2) *Number of animals.* At least eight animals of each sex should be used at each dose level and should be designated for behavioral testing. If interim sacrifices are planned, the number should be increased by the number of animals scheduled to be sacrificed before the end of the study. Animals shall be randomly assigned to treatment and control groups.

(3) *Control groups.* (i) A concurrent ("sham" exposure or vehicle) control

group is required. Subjects shall be treated in the same way as for an exposure group except that administration of the test substance is omitted.

(ii) Concurrent or historic data from the laboratory performing the testing shall provide evidence of the ability of the procedures used to detect major neurotoxic endpoints such as limb weakness or paralysis (e.g., acrylamide), CNS stimulation (e.g., $\beta$, $\beta$ '-iminodiproprionitrile) autonomatic signs (e.g., physostigmine).

(iii) A satellite group may be treated with the high dose level for the duration of exposure and observed for reversibility, persistence, or delayed occurrence of toxic effects for a post-treatment period of appropriate duration, normally not less than 28 days.

(4) *Dose levels and dose selection.* At least 3 doses, equally spaced on a log scale (e.g., ½ log units) over a range of at least 1 log unit shall be used in addition to a zero dose or vehicle administration. The data should be sufficient to produce a dose-effect curve.

(i) The highest dose shall produce (A) clear behavioral effects or (B) life-threatening toxicity.

(ii) The data from the lower doses must show either (A) graded dose-dependent effects at 2 dose levels or (B) no effects at 2 dose levels, respectively.

(5) *Duration and frequency of exposure.* The duration and frequency of exposure will be specified in the test rule.

(6) *Route of exposure.* The test substance shall be administered by the route specified in the test rule. This route will usually be the one most closely approximating the expected route of human exposure. The exposure potocol shall conform to that outlined in the appropriate acute or subchronic toxicity study guideline under Subpart B or Subpart C of this part.

(7) *Combined protocol.* Subjects used for other toxicity studies may be used if none of the requirements of either study are violated by the combination.

(8) *Study conduct.* (i) All animals in a given study should be observed carefully by trained technicians who are blind with respect to the animals' treatments. Standard procedures to

minimize observer variability sha
followed. Where possible, it is ad
ble that the same observer be us
evaluate the animals in a given s
If this is not possible, some demon
tion of inter-observer reliability
quired. All animals should be obse
prior to initiation of exposure. S
quent observations should be
with sufficent frequency to ensur
detection of behavioral and/or n
logic abnormalities, if present. At
imum, observations at 1 hour, 6 h
24 hours, 7 days, and 14 days
monthly thereafter are recommen
In a subchronic study, subseque
the first exposure all observa
should be made before the daily e
sure. The animals should be rem
from the home cage to a stan
arena for observation. Effort sh
be made to ensure that variation
the test conditions are minimal
are not systematically related to t
ment. Among the variables that
affect behavior are sound level,
perature, humidity, lighting, o
time of day, and environmental
tractions. Explicit, operationally
fined scales for each function sh
be used. The development of obje
quantitative measures of the obse
tional endpoints specified is enc
aged.

(ii) The following is a minimal li
observations that shall be noted:

(A) Any unusual responses with
spect to body position, activity l
coordination of movement, and gai

(B) Any unusual or bizarre beha
including, but not limited to, h
flicking, head searching, compul
biting or licking, self-mutilation,
cling, and walking backwards.

(C) The presence of:

(*1*) Convulsions.

(*2*) Tremors.

(*3*) Increased levels of lacrima
and/or red-colored tears.

(*4*) Increased levels of salivation.

(*5*) Piloerection.

(*6*) Pupillary dilation or constrict

(*7*) Unusual respiration (shallow,
bored, dyspneic, gasping, and retch
and/or mouth breathing.

(*8*) Diarrhea.

(*9*) Excessive or diminished ur
tion.

(*10*) Vocalization.

(D) Forelimb/hindlimb grip strength. The procedure described by Meyer et al. (1979), under paragraph (f)(9) of this section is recommended.

(E) Sensory function. A simple assessment of sensory function (vision, audition, pain perception) shall be made. Marshall et al. (1971) under paragraph (f)(8) of this section have described a neurologic exam for this purpose; these procedures are also discussed by Deuel (1977), under paragraph (f)(4) of this section. Irwin (1968) under paragraph (f)(7) of this section described a number of reflex tests intended to detect gross sensory deficits, including the visual placing response, Preyer reflex, and tail pinch. Many procedures have been developed for assessing pain perception (e.g., Ankier, 1974 under paragraph (f)(1) of this section; D'Amour and Smith 1941 under paragraph (f)(3) of this section; Evans 1971 under paragraph (f)(6) of this section).

(e) *Data reporting and evaluation.* In addition to the reporting requirements specified under 40 CFR Part 792 Subpart J the final test report must include the following information.

(1) *Description of system and test methods.* (i) A detailed description of the procedures used to standardize observation, including the arena and operational definitions for scoring observations.

(ii) Positive control data from the laboratory performing the test that demonstrate the sensitivity of the procedures being used. Historic data may be used if all aspects of the experimental protocol are the same, including personnel.

(2) *Results.* The following information must be arranged by test group dose level.

(i) In tabular form, data for each animal must be provided showing:

(A) Its identification number.

(B) Its body weight and score on each sign at each observation time, the time and cause of death (if appropriate).

(ii) Summary data for each group must include:

(A) The number of animals at the start of the test.

(B) The number of animals showing each observation score at each observation time.

(C) The percentage of animals showing each abnormal sign at each observation time.

(D) The mean and standard deviation for each continuous endpoint at each observation time.

(3) *Evaluation of data.* The findings of a functional observational battery should be evaluated in the context of preceding and/or concurrent toxicity studies and any correlative histopathological findings. The evaluation shall include the relationship between the doses of the test substance and the presence or absence, incidence and severity, of any neurotoxic effects. The evaluation should include appropriate statistical analyses. Choice of analyses should consider tests appropriate to the experimental design and needed adjustments for multiple comparisons.

(f) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Ankier, S.I. "New hot plate tests to quantify antinociceptic and narcotic antagonist activities," *European Journal of Pharmacology,* 27: 1-4 (1974).

(2) Coughenour, L.L., McLean, J.R. and Parker, R.B. "A new device for the rapid measurement of impaired motor function in mice," *Pharmacology, Biochemistry and Behavior, 6:* 351-353 (1977).

(3) D'Amour, F.E., Smith, D.L. "A method for determining loss of pain sensation," *Journal of Pharmacology and Experimental Therapeutics, 72:* 74-79 (1941).

(4) Deuel, R.K. "Determining sensory deficits in animals," *Methods in Psychobiology* Ed. Myers R.D. (New York: Academic Press, 1977) pp. 99-125.

(5) Edwards, P.M., Parker, V.H. "A simple, sensitive and objective method for early assessment of acrylamide neuropathy in rats," *Toxicology and Applied Pharmacology, 40:* 589-591 (1977).

(6) Evans, W.O. "A new technique for the investigation of some analgesic drugs on reflexive behavior in the rat," *Psychopharmacologia, 2:* 318-325 (1961).

(7) Irwin, S. "Comprehensive observational assessment: Ia. A systematic quantitative procedure for assessing the behavioral and physiologic state of the mouse," *Psychopharmacologia, 13:* 222-257 (1968).

(8) Marshall, J.F., Turner, B.H., Teitlbaum, P. "Sensory neglect produced by lateral hypothalamic damage," *Science, 174:* 523-525 (1971).

(9) Meyer, O.A., Tilson, H.A., Byrd, W.C., Riley, M.T. "A method for the routine assessment of fore- and hindlimb grip strength of rats and mice," *Neurobehavioral Toxicology, 1:* 233-236 (1979).

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### § 798.6200 Motor activity.

(a) *Purpose*—(1) *General.* In the assessment and evaluation of the toxic characteristics of a substance, determination of the effects of administration of the substance on motor activity is useful when neurotoxicity is suspected.

(2) *Acute Motor Activity Test.* The purpose of the acute motor activity test is to examine changes in motor activity occurring over a range of acute exposure levels. These changes may then be evaluated in the context of changes occurring in other organ systems. This test is an initial step in determining the potential of a substance to produce acute neurotoxicity and may be used to screen members of a class of substances for known neurotoxicity, and/or to establish a dosage regimen prior to the initiation of subchronic neurotoxicity testing.

(3) *Subchronic Motor Activity Test.* The purpose of the subchronic motor activity test is to determine whether the repeated administration of a suspected neurotoxicant results in changes in motor activity. These changes may be evaluated in the context of changes occurring in other organ systems. This test is an initial step in determining the potential of a substance to produce subchronic neurotoxicity.

(b) *Definitions.* (1) Neurotoxicity is the adverse effect on the structure or function of the central and/or peripheral nervous system related to exposure to a chemical substance.

(2) Motor activity is any movement of the experimental animal.

(3) A toxic effect is an adverse change in the structure or function of an experimental animal as a result of exposure to a chemical substance.

(c) *Principle of the test method.* The test substance is administered to several groups of experimental animals, one dose being used per group. Measurements of motor activity are made. The exposure levels at which significant changes in motor activity are produced are compared to those levels which produce toxic effects not originating in the central and/or peripheral nervous system.

(d) *Test procedures*—(1) *Animal selection*—(i) *Species and strain.* Testing shall be performed in a laboratory rat or mouse. The choice of species should take into consideration such factors as the comparative metabolism of the chemical and species sensitivity to the toxic effects of the test substance, as evidenced by the results of other studies, the potential for combined studies, and the availability of other toxicity data for the species.

(ii) *Age.* Young adult animals (at least 42 days old for rat or mouse) should be used.

(iii) *Sex.* (A) Equal numbers of animals of each sex are required for each dose level for the motor activity test.

(B) The females shall be nulliparous and nonpregnant.

(2) *Number of animals.* Animals shall be randomly assigned to test and control groups. Each test or control group must be designed to contain a sufficient number of animals at the completion of the study to detect a 40 percent change in activity of the test groups relative to the control group with 90 percent power at the 5 percent level. For most designs, calculations can be made according to Dixon and Massey (1957) under paragraph (f)(1 of this section, Neter and Wasserman (1974) under paragraph (f)(5) of this section, Sokal and Rohlf (1969) unde paragraph (f)(9) of this section, o Jensen (1972) under paragraph (f)(2 of this section.

(3) *Control groups.* (i) A concurren control group is required. This grou must be an untreated group, or, if vehicle is used in administering th

test substance, a vehicle control group. If the toxic properties of the vehicle are not known or cannot be made available, both untreated and vehicle control group are required.

(ii) Positive control data are required to demonstrate the sensitivity and reliability of the activity measuring device and testing procedure. These data should demonstrate the ability to detect increases or decreases in activity and to generate a dose-effect curve or its equivalent using three values of the dose or equivalent independent variable. A single administration of the dose (or equivalent) is sufficient. It is recommended that chemical exposure be used to collect positive control data. Positive control data shall be collected at the time of the test study unless the laboratory can demonstrate the adequacy of historical data for this purpose.

(iii) A satellite group may be treated with the high dose level for 90 days and observed for reversibility, persistence or delayed occurrence of toxic effects for a post-treatment period of appropriate length, normally not less than 28 days.

(4) *Dose levels and dose selection.* At least 3 doses, equally spaced on a log scale (e.g., ½ log units) over a range of at least 1 log unit shall be used in addition to a zero dose or vehicle administration. The data should be sufficient to produce a dose-effect curve.

(i) The highest dose shall produce (A) clear effects on motor activity or (B) life-threatening toxicity.

(ii) The data from the lower doses must show either (A) graded dose-dependent effects at 2 dose levels or (B) no effects at 2 dose levels, respectively.

(5) *Duration of testing.* The duration of exposure will be specified in the test rule.

(6) *Route of administration.* The test substance shall be administered by the method specified in the test rule. This will usually be the route most closely approximating the route of human exposure. The exposure protocol shall conform to that outlined in the appropriate acute or subchronic toxicity study guideline.

(7) *Combined protocol.* The tests described herein may be combined with any other toxicity study, as long as none of the requirements of either are violated by the combination.

(8) *Study conduct*—(i) *General.* Motor activity must be monitored by an automated activity recording apparatus. The device used must be capable of detecting both increases and decreases in activity, i.e. baseline activity as measured by the device must not be so low as to preclude decreases nor so high as to preclude increases. Each device shall be tested by standard procedure to ensure, to the extent possible, reliability of operation across devices and across days for any one device. In addition, treatment groups must be balanced across devices. Each animal shall be tested individually. The test session shall be long enough for motor activity to approach asymptotic levels by the last 20 percent of the session for most treatments and animals. All sessions should have the same duration. Treatment groups shall be counter-balanced across test times. Effort should be made to ensure that variations in the test conditions are minimal and are not systematically related to treatment. Among the variables which can affect motor activity are sound level, size and shape of the test cage, temperature, relative humidity, lighting conditions, odors, use of home cage or novel test cage and environmental distractions. Tests shall be executed by an appropriately trained individual.

(ii) *Acute.* Testing shall be timed to include the time of peak signs.

(iii) *Subchronic.* All animals shall be tested prior to initiation of exposure and at 30 ±2, 60 ±2 and 90 ±2 days during the exposure period. Testing shall occur prior to the daily exposure. Animals shall be weighed on each test day and at least once weekly during the exposure period.

(e) *Data reporting and evaluation.* In addition to the reporting requirements specified under 40 CFR Part 792, Subpart J the final test report must include the following information:

(1) *Description of system and test methods.* (i) Positive control data from the laboratory performing the test which demonstrate the sensitivity of the procedure being used.

(ii) Procedures for calibrating and assuring the equivalence of devices and balancing treatment groups.

(2) *Results.* The following information must be arranged by test group (dose level).

(i) In tabular form, data must be provided showing for each animal:

(A) Its identification number.

(B) Body weight, total session activity counts, and intrasession subtotals for each date measured.

(ii) Group summary data should also be reported.

(3) *Evaluation of data.* An evaluation of the test results (including statistical analysis comparing total activity counts at the end of exposure of treatment vs control animals must be made and supplied. This submission must include dose-effect curves for motor activity expressed as activity counts.

(f) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Dixon, W.J., Massey, E.J. *Introduction to Statistical Analysis* 2nd Ed. (New York: McGraw-Hill, 1957).

(2) Finger, F.W. "Measuring behavioral activity," *Methods in Psychobiology* Vol. 2. Ed. R.D. Myers (New York: Academic, 1972) pp. 1-19.

(3) Jensen, D.R. "Some simultaneous multivariate procedures using Hotelling's $T^2$ Statistics," *Biometrics,* 28:39-53 (1972).

(4) Kinnard, E.J. and Watzman, N. "Techniques utilized in the evaluation of psychotropic drugs on animals activity," Journal of Pharmaceutical Sciences, 55:995-1012 (1966).

(5) Neter, J. and Wasserman, W. *Applied Linear Statistical Models.* Homewood, Richard D. Irwin, Inc., 1974.

(6) Reiter, L.E. "Use of activity measures in behavioral toxicology," *Environmental Health Perspectives,* 26:9-20 (1978).

(7) Reiter, L.W. and MacPhail, R.C. "Motor Activity: A survey of methods with potential use in toxicity testing," *Neurobehavioral Toxicology,* 1: Suppl. 1, 53-66 (1979).

(8) Robbins, T.W. "A critique of the methods available for the measurement of spontaneous motor activity,"
*Handbook of Psychopharmacol*
Vol. 7. Eds. Iversen, L.L., Iversen, I
Snyder, S.H. (New York: Plen
1977) pp. 37-82.

(9) Sokal, R.P. and Rohlf, E.J. *Bi*
*etry.* (San Francisco: W.H. Free
and Co., 1969).

[50 FR 39397, Sept. 27, 1985, as amende
52 FR 19082, May 20, 1987]

### § 798.6400 Neuropathology.

(a) *Purpose.* The techniques in
guideline are designed to develop
on morphologic changes in the n
ous system for chemical substa
and mixtures subject to such tes
under the Toxic Substances Con
Act. The data will detect and cha
terize morphologic changes, if
when they occur, and determine a
effect level for such changes. Ne
pathological evaluation should
complemented by other neurotox
studies, e.g. behavioral and neuro
siological studies. Neuropatholo
evaluation may be done follo
acute, subchronic or chronic expos

(b) *Definition.* Neurotoxicity
neurotoxic effect is an adverse cha
in the structure or function of
nervous system following exposur
a chemical agent.

(c) *Principle of the test method.*
test substance is administered to se
al groups of experimental animals,
dose being used per group. The
mals are sacrificed and tissues in
nervous system are examined gro
and prepared for microscopic exam
tion. Starting with the highest do
level, tissues are examined under
light microscope for morpho
changes, until a no effect level is
termined. In cases where light mi
copy has revealed neuropathology,
no effect level may be confirme
electron microscopy.

(d) *Test procedure*—(1) *Animal s*
*tion*—(i) *Species and strain.* Tes
shall be performed in the species b
used in other tests for neurotox
This will generally be the labora
rat. The choice of species shall
into consideration such factors as
comparative metabolism of the ch
cal and species sensitivity to the
effects of the test substance, as
denced by the results of other stu

the potential for combined studies, and the availability of other toxicity data for the species.

(ii) *Age.* Animals shall be young adults (150-200 gm for rats) at the start of exposure.

(iii) *Sex.* Both sexes shall be used unless it is demonstrated that one sex is refractory to the effects.

(2) *Number of Animals.* A minimum of six animals per group shall be used. The tissues from each animal shall be examined separately. It is recommended that ten animals per group be used.

(3) *Control Groups.* (i) A concurrent control group(s) is (are) required. This group must be an untreated control group or, if a vehicle is used in administering the test substance, a vehicle control group. If the vehicle used has a known or potential toxic property, both untreated and vehicle control groups are required.

(ii) A satellite group of animals may be treated with the high level for 90 days and observed for reversibility, persistence, or delayed occurrence of toxic effects for a post-treatment period of appropriate length; normally not less than 28 days.

(4) *Dose Levels and Dose Selection.* At least 3 doses, equally spaced on a log scale (e.g., ½ log units) over a range of at least 1 log unit shall be used in addition to a zero dose or vehicle administration. The data should be sufficient to produce a dose-effect curve.

(i) The highest dose shall produce (A) clear behavioral effects or (B) life-threatening toxicity.

(ii) The data from the lower doses must show either (A) graded dose-dependent effects at two dose levels or (B) no effects at two dose levels, respectively.

(5) *Duration of testing.* The exposure duration will be specified in the test rule. This will generally be 90 days exposure.

(6) *Route of administration.* The test substance shall be administered by a route specified in the test rule. This will generally be the route most closely approximating the route of human exposure. The exposure protocol shall conform to that outlined in the appropriate acute or subchronic toxicity guideline.

(7) *Combined protocol.* The tests described herein may be combined with any other toxicity study, as long as none of the requirements of either are violated by the combination.

(8) *Study conduct*—(i) *Observation of animals.* All toxicological (e.g., weight loss) and neurological signs (e.g., motor disturbance) shall be recorded frequently enough to observe any abnormality, and not less than weekly.

(ii) *Sacrifice of animals*—(A) *General.* The goal of the techniques outlined for sacrifice of animals and preparation of tissues is preservation of tissues morphology to simulate the living state of the cell.

(B) *Perfusion technique.* Animals shall be perfused *in situ* by a generally recognized technique. For fixation suitable for light or electronic microscopy, saline solution followed by buffered 2.5 percent glutaraldehyde or buffered 4.0 percent paraformaldehyde, is recommended. While some minor modifications or variations in procedures are used in different laboratories, a detailed and standard procedure for vascular perfusion may be found in the text by Zeman and Innes (1963) under paragraph (f)(7) of this section, Hayat (1970) under paragraph (f)(3) of this section, and by Spencer and Schaumburg (1980) under paragraph (f)(6) of this section. A more sophisticated technique is described by Palay and Chan-Palay (1974) under paragraph (f)(4) of this section.

(C) *Removal of brain and cord.* After perfusion, the bonystructure (cranium and vertebral column) shall be exposed. Animals shall then be stored in fixative-filled bags at 4°C for 8-12 hours. The cranium and vertebral column shall be removed carefully by trained technicians without physical damage of the brain and cord. Detailed dissection procedures may be found in the text by Palay and Chan-Palay (1974) under paragraph (f)(4) of this section. After removal, simple measurement of the size (length and width) and weight of the whole brain (cerebrum, cerebellum, pons-medulla) shall be made. Any abnormal coloration or discoloration of the brain and cord shall also be noted and recorded.

(D) *Sampling.* Unless a given test rule specifies otherwise, cross-sections of the following areas shall be examined: The forebrain, the center of the cerebrum, the midbrain, the cerebellum and pons, and the medulla oblongata; the spinal cord at cervical and lumbar swelling ($C_3$-$C_6$ and $L_1$-$L_4$); Gasserian ganglia, dorsal root ganglia ($C_3$-$C_6$, $L_1$-$L_4$), dorsal and ventral root fibers ($C_3$-$C_6$, $L_1$-$L_4$), proximal sciatic nerve (mid-thigh and sciatic notch), sural nerve (at knee), and tibial nerve (at knee). Other sites and tissue elements (e.g.. gastrocnemius muscle) should be examined if deemed necessary. Any observable gross changes shall be recorded.

(iii) *Specimen storage.* Tissue samples from both the central and peripheral nervous system shall be further immersion fixed and stored in appropriate fixative (e.g., 10 percent buffered formalin for light microscopy; 2.5 percent buffered gluteraldehyde or 4.0 percent buffered paraformaldehyde for electron microscopy) for future examination. The volume of fixative versus the volume of tissues in a specimen jar shall be no less than 25:1. All stored tissues shall be washed with buffer for at least 2 hours prior to further tissue processing.

(iv) *Histopathology examination.* (A) *Fixation.* Tissue specimens stored in 10 percent buffered formalin may be used for this purpose. All tissues must be immersion fixed in fixative for at least 48 hours prior to further tissue processing.

(B) *Dehydration.* All tissue specimens shall be washed for at least 1 hour with water or buffer, prior to dehydration. (A longer washing time is needed if the specimens have been stored in fixative for a prolonged period of time.) Dehydration can be performed with increasing concentration of graded ethanols up to absolute alcohol.

(C) *Clearing and embedding.* After dehydration, tissue specimens shall be cleared with xylene and embedded in paraffin or paraplast. Multiple tissue specimens (e.g. brain, cord, ganglia) may be embedded together in one single block for sectioning. All tissue blocks shall be labelled showing at
least the experiment number, an
number, and specimens embedded.

(D) *Sectioning.* Tissue sections,
6 microns in thickness, shall be
pared from the tissue blocks
mounted on standard glass slides.
recommended that several additi
sections be made from each bloc
this time for possible future needs
special stainings. All tissue blocks
slides shall be filed and stored in p
erly labeled files or boxes.

(E) *Histopathological techniques*
though the information available f
given chemical substance may dic
test-rule specific changes, the fol
ing general testing sequence is
posed for gathering histopatholo
data:

(*1*) *General staining.* A gen
staining procedure shall be perfor
on all tissue specimens in the hig
treatment group. Hematoxylin
eosin (H&E) shall be used for this
pose. The staining shall be differe
ated properly to achieve bluish nu
with pinkish background.

(*2*) *Special stains.* Based on the
sults of the general staining, sele
sites and cellular components sha
further evaluated by the use of sp
ic techniques. If H&E screening
not provide such information, a
tery of stains shall be used to as
the following components in all ap
priate required samples: neur
body (e.g.. Einarson's gallocyar
axon (e.g., Bodian), myelin sh
(e.g.. Kluver's Luxol Fast Blue)
neurofibrils (e.g.. Bielchosky). In a
tion, peripheral nerve fiber tea
shall be used. Detailed staining m
odology is available in standard hi
technological manuals such as A
(1968) under paragraph (f)(1) of
section, Ralis et al. (1973) under p
graph (f)(5) of this section, and Ch
(1979) under paragraph (f)(2) of
section. The nerve fiber teasing t
nique is discussed in Spencer
Schaumberg (1980) under paragr
(f)(6) of this section. A section
normal tissue shall be included in e
staining to assure that adequate st
ing has occurred. Any changes sha
noted and representative photogra
shall be taken. If a lesion(s) is
served, the special techniques sha
repeated in the next lower treatn

group until no further lesion is detectable.

(*3*) *Alternative technique.* If the anatomical locus of expected neuro-pathology is well-defined, epoxy-embedded sections stained with toluidine blue may be used for small sized tissue samples. This technique obviates the need for special stains for cellular components. Detailed methodology is available in Spencer and Schaumberg (1980) under paragraph (f)(6) of this section.

(*4*) *Electron microscopy.* Based on the results of light microscopic evaluation, specific tissue sites which reveal a lesion(s) shall be further evaluated by electron microscopy in the highest treatment group which does not reveal any light microscopic lesion. If a lesion is observed, the next lower treatment group shall be evaluated until no significant lesion is found. Detailed methodology is available in Hayat (1970) under paragraph (f)(3) of this section.

(F) *Examination—(1) General.* All stained microscopic slides shall be examined with a standard research microscope. Examples of cellular alterations (e.g., neuronal vacuolation, degeneration, and necrosis) and tissue changes (e.g., gliosis, leukocytic infiltration, and cystic formation) shall be recorded and photographed.

(*2*) *Electron microscopy.* Since the size of the tissue samples that can be examined is very small, at least 3 to 4 tissue blocks from each sampling site must be examined. Tissue sections must be examined with a transmission electron microscope. Three main categories of structural changes must be considered:

(*i*) *Neuronal body.* The shape and position of the nucleus and nucleolus as well as any change in the chromatin patterns shall be noted. Within the neuronal cytoplasm, cytoplasmic organelles such as mitochondria, lysosomes, neurotubules, neurofilaments, microfilaments, endoplasmic reticulum and polyribosomes (Nissl substance), Golgi complex, and secretory granules shall be examined.

(*ii*) *Neuronal processes.* The structural integrity or alterations of dendrites, axons (myelinated and unmyelinated), myelin sheaths, and synapses shall be noted.

(*iii*) *Supporting cells.* Attention must also be paid to the number and structural integrity of the neuroglial elements (oligodendrocytes, astrocytes, and microglia) of the central nervous system, and the Schwann cells, satellite cells, and capsule cells of the peripheral nervous system. Any changes in the endothelial cells and ependymal lining cells shall also be noted whenever possible. The nature, severity, and frequency of each type of lesion in each specimen must be recorded. Representative lesions must be photographed and labeled appropriately.

(e) *Data collection, reporting, and evaluation.* In addition to information meeting the requirements stated under 40 CFR Part 792 Subpart J, the following specific information shall be reported:

(1) *Description of test system and test methods.* A description of the general design of the experiment shall be provided. This shall include a short justification explaining any decisions where professional judgment is involved such as fixation technique and choice of stains.

(2) *Results.* All observations shall be recorded and arranged by test groups. This data may be presented in the following recommended format:

(i) *Description of signs and lesions for each animal.* For each animal, data must be submitted showing its identification (animal number, treatment, dose, duration), neurologic signs, location(s) nature of, frequency, and severity of lesion(s). A commonly-used scale such as 1+, 2+, 3+, and 4+ for degree of severity ranging from very slight to extensive may be used. Any diagnoses derived from neurologic signs and lesions including naturally occurring diseases or conditions, should also be recorded.

(ii) *Counts and incidence of lesions, by test group.* Data shall be tabulated to show:

(A) The number of animals used in each group, the number of animals displaying specific neurologic signs, and the number of animals in which any lesion was found;

(B) The number of animals affected by each different type of lesion, the average grade of each type of lesion, and the frequency of each different type and/or location of lesion.

(iii) *Evaluation of data.* (A) An evaluation of the data based on gross necropsy findings and microscopic pathology observations shall be made and supplied. The evaluation shall include the relationship, if any, between the animal's exposure to the test substance and the frequency and severity of the lesions observed.

(B) The evaluation of dose-response, if existent, for various groups shall be given, and a description of statistical method must be presented. The evaluation of neuropathology data should include, where applicable, an assessment in conjunction with other neurotoxicity studies performed (eg. electrophysiological, behavioral, neurochemical).

(f) *References.* For additional background information on this test guideline the following references should be consulted:

(1) AFIP. *Manual of Histologic Staining Methods.* (New York: McGraw-Hill (1968).

(2) Chang, L.W. *A Color Atlas and Manual for Applied Histochemistry.* (Springfield, IL: Charles C. Thomas, 1979).

(3) Hayat, M.A. "Vol. 1. Biological applications," *Principles and techniques of electron microscopy.* (New York: Van Nostrand Reinhold, 1970)

(4) Palay S.L., Chan-Palay, V. *Cerebellar Cortex: Cytology and Organization.* (New York: Springer-Verlag, 1974).

(5) Ralis, H.M., Beesley, R.A., Ralis, Z.A. *Techniques in Neurohistology.* (London: Butterworths, 1975).

(6) Spencer, P.S., Schaumburg, H.H. (eds). *Experimental and Clinical Neurotoxicology.* (Baltimore: Williams and Wilkins, 1980).

(7) Zeman, W., JRM Innes, J.R.M. *Craigie's Neuroanatomy of the Rat.* (New York: Academic, 1963).

[50 FR 39397, Sept. 27, 1985, as amended at 52 FR 19082, May 20, 1987]

## § 798.6450 NTE neurotox assay.

(a) *Purpose.* (1) A variety of organophosphorus (O-P) compounds cause a
type of delayed neurotox
(OPIDN) in which inhibition of th
terase activity of a protein called
rotoxic esterase (NTE) in neural ti
is the primary biochemical corre
and predictor. This guideline will
scribe the conduct of an assay
measurement of the inhibition of
in the brain or spinal cord of ani
exposed to O-P. It is based on
method described by Johnson (1
1977, 1982) under paragraphs (f
(3), and (4) of this section and qu
those sources directly. Other meth
for performing the assay have b
described by Sprague et al. (1
under paragraph (f)(7) of this sect
Soliman et al. (1982) under paragr
(f)(6) of this section and Caroldi
Lotti (1982) under paragraph (f)(1
this section for peripheral ne
These and other assays are revie
by Johnson (1982) under paragr
(f)(4) of this section and Johnson
Richardson (1983) under paragr
(f)(5) of this section. This biochem
test is intended as an adjunct to a
havioral and pathological study
hens.

(2) NTE measurements provide
jective quantitative data on the
step in initiation of OPIDN. T
they can be a great help in evalua
studies where the more poorly qua
fied behavioral or pathological
may be equivocal, or where the
quacy of a negative study is at is
The second step necessary is "agi
of the phosphorylated NTE (the
of an R group resulting in a negati
charged substituent bound to N
Not all O-P that inhibit NTE, t
cause OPIDN, but all neuropathic
inhibit NTE.

(3) Thus, the NTE assay is a va
ble adjunct to but not a complete
placement for in-vivo testing. John
(1982) (under paragraph (f)(4) of
section has proposed a detailed tes
scheme for combining the NTE a
with behavioral and histopatholog
studies in hens. Such combined tes
can be faster, more accurate, and
expensive than the current EPA
proach. OTS encourages tes
schemes that combine this assay w
in-vivo studies.

(b) *Definitions.* (1) Organop
phorus induced delayed neurotox

(OPIDN) is a neurological syndrome in which limb weakness and upper motor neuron spasticity are the predominant clinical signs and distal axonopathy of peripheral nerve and spinal cord are the correlative pathological signs. Clinical signs and pathology first appear between 1 and 2 weeks following exposure which normally inhibits greater than 80 percent of NTE (For O-Ps that "age").

(2) Neurotoxic target esterase (NTE) is a membrane-bound neural protein that hydrolyzes phenyl valerate and is highly correlated with the initiation of OPIDN. NTE activity is operationally defined as the phenyl valerate hydrolytic activity resistant to paraoxon but sensitive to mipafox or neuropathic O-P ester inhibition.

(c) *Principle of the test method.* The test method is a differential assay of the ability of neural tissue, following O-P exposure, to selectively hydrolyze a phenyl valerate substrate. The principle of the assay is first, to determine the amount of hydrolysis that occurs in the presence of a non-neurotoxic inhibitor, paraoxon, (a), which is intended to occupy irrelevant sites. Second, we determine the activity in the presence of paraoxon and a known neuropathic inhibitor, mipafox, (b). NTE activity is the difference between (a) and (b), that is, the proportion of activity inhibited only by mipafox. Thus, the "mipafox site" is already occupied following exposure to a neuropathic O-P ester and the activity of (b) is therefore reduced.

(d) *Testing procedure.* Because this guideline is intended as an adjunct to an in-vivo study, only those aspects of study conduct specific to the assay will be described.

(1) *Animal selection.* The adult domestic laying hen is recommended. Standard size breeds and strains should be employed.

(2) *Number of animals.* (i) At least four hens/dose or control group should be used.

(ii) It is recommended that at least two hens be dosed with a known neuropathic O-P as a positive control. Tri-o-tolyl phosphate (TOCP) or diisopropyl phosphorofluoridate (DFP) can be used but TOCP is recommended because of its low acute toxicity.

(3) *Materials.* To conduct this assay, you will need paraoxon (diethyl 4-nitrophenyl phosphate), mipafox (N, N'-diisopropylphosphorodiamido fluoridate), and phenyl valerate. Johnson (1977) under paragraph (f)(3) of this section includes a detailed description of the steps necessary for the synthesis, purification, and storage of these chemicals. Paraoxon can be obtained commercially.

(4) *Study conduct.* (i) The assay should be performed on the whole brain and spinal cord of subjects sacrificed 24 hours following acute exposure, and at regular intervals, e.g., weekly, during repeated exposure. The last measurement should follow the last exposure by 24 or 48 hours. Each assay should be performed in duplicate.

(ii) The assay has four stages: Preparation of tissue; differential preincubation; hydrolysis of substrate; and measurement of product. The quotations that follow are from Johnson (1977) as corrected or modified in Johnson (1982). His is the best known method for conduct of this assay. Other acceptable methods have been used. They primarily involve minor technical modification (Sprague et al. 1981 under paragraph (f)(7) of this section and Soliman et al. 1982 under paragraph (f)(6) of this section). Testers are encouraged to discuss planned methods with EPA prior to conducting testing for approval.

(A) *Preparation of tissue.* "Normal or dosed birds are killed by cervical dislocation. The head is cut off and the whole brain removed and cooled in ice-cold buffer (50 mM Tris/0.2 mM EDTA adjusted to pH 8.0 at 25° with HCL). Meninges and blood vessels are rapidly removed and the brain is blotted dry, weighed, and homogenized thoroughly in ice-cold buffer (6.5 ml/g) using a high-speed rotating perspex pestle with not more than 0.25 mm difference in diameter between pestle and tube. The homogenate is then diluted to 1 g/65 ml for assay."

(B) *Differential preincubation.* "Paired samples of homogenate (equivalent to about 6.0 mg tissue) are pre-incubated in Tris/EDTA buffer pH 8 at 37° for exactly 20 minutes with paraoxon (40 to 100 uM) plus either (a) buffer or (b) mipafox (50uM) in a final volume of 2 ml." This step is essential.

(C) *Hydrolysis of substrate.* "After preincubation, dispersion (2ml) of phenyl valerate is added and the incubation is continued for exactly 15 minutes. The dispersion is prepared by adding a solution of Triton X-100 (0.03 percent in water) (30 vol) to a solution of phenyl valerate (15 or 30 mg/ml) in redistilled dimethylformamide (1 vol) and mixing thoroughly (by swirling): other solvents give less satisfactory dispersions. Reaction is stopped by adding 2 ml of sodium dodecyl sulphate (1% w/v) in buffer containing 4-aminoantipyrine (otherwise known as 4-aminophenazone) (0.25 percent)."

(D) *Measurement of product.* This assay is based on the colorimetric detrimination of liberated phenol.

(*1*) "The coupling of phenol liberated in the assay with the aminoantipyrine may be performed at any convenient time after quenching the enzyme: 1 ml of $K_3Fe(CN)_6$ (0.4 percent in water) is added and the stable red colour is read at 510 nm."

(*2*) "A non-tissue blank, kept to 10 percent of the "B" (paraoxon tube) value by maintaining the substrate phenol fee, should be included in each group of assay tubes. Typical control absorbance values would be 0.8 for paraoxon, 0.35 for paraoxon and mipafox and 0.07 for the blank. Colour development takes (1-2 min) in solutions stopped with sodium dodecyl sulphate. The extinction coefficient of phenol under these conditions is 13,900. NTE activity is represented by the difference in absorbance obtained from samples incubated under conditions (a) and (b) respectively."

(*3*) "To clarify the incubation media when NTE activity of spinal cord homogenates is assayed, (up to 16 mg wet weight/tube), increase sodium dodecyl sulphate concentration to 2 percent. Under standard conditions NTE hydrolyzes about 2400 umol of substrate/min/g of cortex, 550 for spinal cord, and 100 for sciatic nerve."

(e) *Data reporting and evaluation*—(1) *Test report.* In addition to the reporting requirements specified in the EPA Good Laboratory Practice Standards under 40 CFR Part 792, Subpart J. The final test report must inclu the following information:

(i) Neurotoxic esterase data inclu ing absorbance values for each subj tested.

(ii) Indication of whether each su ject survived to sacrifice or time death.

(iii) Data from control animals a blank samples.

(iv) Statistical evaluation of result

(2) *Evaluation of results.* (i) Resu should be evaluated in terms t extent of inhibition as a function treatment and dose, comparison to hibition of acetylcholinesterase (A E) where available, lethal potency, a data on blank samples and cont group results.

(ii) Results on NTE should be co pared to and evaluated with behavi al and histopathological data. Seque tial data from repeated exposu studies should also be evaluated evidence that the NTE level has p teaued.

(f) *References.* For additional ba ground information on this test gui line the following references should consulted:

(1) Caroldi, S., Lotti, M. "Neuroto Esterose in Peripheral Nerve: Ass Inhibition, and Rate of Resynthes *Toxicology and Applied Pharmaco gy,* 62, 498-501 (1982).

(2) Johnson, M.K. "The Dela Neuropathy Caused By Some Orga phosphorus Esters: Mechanism Challenge," *Critical Reviews In To cology,* 3, 289-316 CRC Press, I (1975).

(3) Johnson, M.K. "Improved As of Neurotoxic Esterase for Screen Organophosphates for Delayed Neu toxicity Potential," *Archives of To cology,* 37, 113-115 (1977).

(4) Johnson, M.K. "The Target Initiation of Delayed Neurotoxicity Organophosphorus Esters: Bioche cal Studies and Toxicological Appli tions," Reviews in *Biochemical To cology,* 4. Eds. Hodgson, E., Bend, Philpot, R.M., (Elsevier: New Yo 1982); pp. 141-212.

(5) Johnson, M.K., Richardson, "Biochemical Endpoints: Neuroto Esterase Assay," *Neurotoxicology,* 4 311-320 (1983).

(6) Soliman, S.A., Linder, R., Farmer, J., Curley, A. "Species Susceptibility to Delayed Toxic Neuropathy in relation to in vivo inhibition of Neurotoxic Esterase by Neurotoxic Organophosphorus Esters," *Journal of Toxicology and Environmental Health*, 9, 189-197 (1982).

(7) Sprague, G.L., Sandvik, L.L., Bickford, A.A. "Time course for neurotoxic esterase activity in hens given multiple diisopropyl fluorophosphate injections," *Neurotoxicology*, 2, 523-532 (1981).

## § 798.6500 Schedule-controlled operant behavior.

(a) *Purpose.* (1) In the assessment and evaluation of the potential human health effects of substances, it may be necessary to test for functional neurotoxic effects. Substances that have been observed to produce neurotoxic signs in other toxicity studies (e.g. CNS depression or stimulation), as well as substances with a structural similarity to known neurotoxicants should be evaluated for these effects.

(2) This guideline defines procedures for conducting studies of schedule-controlled operant behavior, one way of evaluating functional neurotoxic effects (Dews, 1972 under paragraph (f)(1) of this section; NAS 1975, 1977, 1982 under paragraphs (f)(4), (5) and (6) of this section). Our purpose is to evaluate the effects of acute and repeated exposures on the rate and pattern of responding under schedules of reinforcement. Operant behavior tests may be used to evaluate many other aspects of behavior (Laties, 1978 under paragraph (f)(3) of this section). Additional tests may be necessary to completely assess the behavioral effects of any substance. Behavioral evaluation should be used in conjunction with neuropathologic evaluation and the evaluation of other toxic effects.

(b) *Definitions*—(1) *Neurotoxicity.* Neurotoxicity or a neurotoxic effect is an adverse change in the structure or function of the nervous system following exposure to a chemical agent. Behavioral toxicity is an adverse change in the functioning of the organism with respect to its environment following exposure to a chemical agent.

(2) *Operant, operant behavior, operant conditioning.* An operant is a class of behavioral responses which change or operates on the environment in the same way. Operant behavior is further distinguished as behavior which is modified by its consequences. Operant conditioning is the experimental procedure used to modify some class of behavior by reinforcement or punishment.

(3) *Schedule of reinforcement.* A schedule of reinforcement specifies the relation between behavioral responses and the delivery of reinforcers, such as food or water (Ferster and Skinner, 1957 under paragraph (f)(2) of this section). For example, a fixed ratio (FR) schedule requires a fixed number of responses to produce a reinforcer (e.g. FR 30). On a fixed interval (FI) schedule, the first response after a fixed period of time is reinforced (e.g. FI 5 minutes).

(c) *Principle of the test method.* Experimental animals are trained to perform under a schedule of reinforcement and measurements of their operant behavior are made. Several doses of the test substance are then administered according to the experimental design (between groups or within subjects) and the duration of exposure (acute or repeated). Measurements of the operant behavior are repeated. A descriptive and statistical evaluation of the data is made to evaluate the nature and extent of any changes in behavior in relation to exposures to the test substance. Comparisons are made between any exposures that influence the behavior and exposures that have neuropathological effects or effects on other targets of the chemical.

(d) *Test procedures*—(1) *Experimental design.* These test procedures may be used to evaluate the behavior of experimental animals receiving either acute or repeated exposures. For acute exposure studies, either within-subject or between groups, experimental designs may be used. For repeated exposure studies, between groups designs should be used, but within subject comparisons (pre-exposure and post-exposure) are recommended and encouraged.

(2) *Animal selection*—(i) *Species*. (A) For most studies, the laboratory mouse or rat is recommended. Standard strains should be used.

(B) Under some circumstances other species may be recommended.

(ii) *Age*. Experimental animals should be young adults. Rats or mice should be at least 14 and 6 weeks old, respectively, prior to exposure.

(iii) *Sex*. (A) Approximately equal numbers of male and female animals are required for each dose level and control group.

(B) Virgin females should be used.

(iv) *Experimental history*. Animals should be experimentally and chemically naive.

(3) *Number of animals*. Six to twelve animals should be exposed to each level of the test substance and/or control procedure. If post exposure effects are examined, a separate group, 6 to 12 additional animals not sacrificed for pathology, will required in subchronic studies.

(4) *Control groups*—(i) *Untreated controls*. A concurrent "sham" exposure or vehicle control group or session (according to the design of the study) is required. The subjects should be treated similarly except that administration of the test substance is omitted.

(ii) *Positive controls*. Positive control data is required to demonstrate that the experimental procedures, under the specific conditions in the testing laboratory, are sensitive to substances known to affect operant behavior. Both increases and decreases in response rate should be demonstrated. Data based on acute exposures will be adequate. Data should be collected according to the same experimental design as that proposed for the test substance. Historical data on the procedure collected in the same species and under the same conditions in the testing laboratory may be acceptable, but the presentation of concurrent control data is strongly encouraged since it provides evidence that the test has remained sensitive.

(5) *Dose levels and dose selection*. At least 3 doses, equally spaced over a log scale (e.g., 10, 30, 100), over a range of at least 1 log unit shall be used in addition to a zero dose or vehicle administration. The data should be sufficient to produce a dose-effect curve.

(i) The highest dose shall produce: (A) Clear behavioral effects; or (B) life-threatening toxicity.

(ii) The data from the lower doses must show either: (A) Graded dose-dependent effects at 2 dose levels; or (B) no effects at 2 dose levels, respectively.

(6) *Duration of exposure*. The duration and frequency of exposure will be specified in the test rule.

(7) *Route of Administration*. The route of administration will also be specified in the test rule and will usually be identical to one of the anticipated or actual routes of human exposure. For some chemicals, another route (e.g. parenteral) may be justified. The exposure protocol should conform to that outlined in the appropriate acute or subchronic toxicity study guideline under Subpart B or Subpart C of this part.

(8) *Study conduct*—(i) *Apparatus*. Behavioral responses and the delivery of reinforcers shall be controlled and monitored by automated equipment located so that its operation does not provide unintended cues or otherwise interfere with the ongoing behavior. Individual chambers should be sound attenuated to prevent disruptions of behavior by external noise. The response manipulanda, feeders, and any stimulus devices should be tested before each session; these devices should periodically be calibrated.

(ii) *Chamber assignment*. Concurrent treatment groups should be balanced across chambers. Each subject should be tested in the chamber to which it is initially assigned.

(iii) *Deprivation and training*. (A) If a nonpreferred positive reinforcer is used, all subjects should be deprived of food until they reach a fixed percentage (e.g. 80 to 90 percent, commonly) of their ad libitum body weight or for a fixed period (e.g., 18 hours) prior to training. Deprivation should be kept constant throughout the study.

(B) Subjects must be trained until they display demonstrable stability in performance across days prior to exposure. One simple and useful criterion is a minimum number of sessions on the schedule and no systematic trend during the 5 days before exposure.

(C) Cumulative records of cumulative responding over time for each animal should be presented to demonstrate that the pattern of responding is representative of that generated by the schedule of reinforcement.

(iv) *Time, frequency, and duration of testing*—(A) *Time of testing.* All experimental animals should be tested at the same time of day and with respect to the time of exposure. For acute studies, testing should be performed when effects are estimated to peak, usually shortly after exposure. For subchronic studies, subjects should be tested prior to daily exposure in order to assess cumulative effects.

(B) *Frequency of testing.* The maintenance of stable operant behavior normally will require regular and frequent (e.g., 5 days a week) testing sessions. Animals should be weighed on each test day.

(C) *Duration of testing.* (*1*) Experimental sessions should be long enough to reasonably see the effects of exposure, but brief enough to be practical. Under most circumstances, a session length of 30-40 minutes should be adequate.

(*2*) If the nature or duration of effects following cessation of repeated exposure are a concern, animals from the high dose group should be tested following exposure for a suitable period of time.

(v) *Schedule selection.* The schedule of reinforcement chosen should generate response rates that may increase or decrease as a function of exposure. Many schedules of reinforcement can do this: a single schedule maintaining a moderate response rate; fixed-interval schedules, which engender a variety of response rates in each interval; or multiple schedules, where different components may maintain high and low response rates.

(e) *Data reporting and evaluation.* In addition to the reporting requirements specified under 40 CFR Part 792, Subpart J the final test report should contain the following information:

(1) *Description of system, test methods, experimental design, and control data.* (i) A description of the experimental chamber, programming equipment, data collection devices, and environmental conditions.

(ii) A description of the experimental design including counterbalancing procedures, and the stability criterion.

(iii) A description and statistical evaluation of positive control and other control data, including standard measures of central tendency, variability, coefficient of variation of response rates, and the slope of the dose-effect curve.

(2) *Results.* (i) Data for each animal should be arranged by test group in tabular form including the animal identification number, body weight, pre-exposure rate of responding, changes in response rate produced by the chemical, and group data for the same variables, including standard measures of central tendency, variability and coefficient of variation.

(ii) A description and statistical evaluation of the test results: With particular reference to the overall statistical procedures (e.g., parametric or nonparametric) dose-effect curve, and calculation of slope. Presentation of calculations is encouraged.

(f) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Dews, P.B. "Assessing the Effects of Drugs," *Methods in Psychobiology,* Vol. 2, Ed., R.D. Myers (New York: Academic Press, 1972) 83-124.

(2) Ferster, C.B. Skinner, B.F. Schedules of Reinforcement. (New York: Appleton-Century-Crofts, 1957).

(3) Laties, V.G. "How Operant Conditioning can Contribute to Behavioral Toxicology," Environmental Health Perspectives, 28: 29-35 (1978).

(4) National Academy of Science. *Principles for Evaluating Chemicals in the Environment.* (Washington, DC: National Academy of Sciences, 1975).

(5) National Academy of Science. *Principles and Procedures for Evaluating the Toxicity of Household Substances.* (Washington, DC: National Academy of Sciences, 1977).

(6) National Academy of Science. "Strategies to determine needs and priorities for toxicity testing," Appendix 3B. *Reference Protocol Guidelines For Neurobehavioral Toxicity Tests.* 2: 123-129 (1982).

**§ 798.6540 Acute delayed neurotoxicity of organophosphorus substances.**

(a) *Purpose.* Organophosophorus substances should be considered as candidates for delayed neurotoxicity studies using the adult hen as the test animal. This test has certain limitations, e.g., in predicting effects from repeated exposures. These limitations may be minimized by conducting an adjunct test in which inhibition and aging of neurotoxic esterase of hen neural tissue are measured.

(b) *Definitions.* Acute delayed neurotoxicity is a prolonged, delayed-onset locomotor ataxia resulting from single administration of the test substance, repeated once if necessary.

(c) *Principle of the test method.* The test substance is administered orally in a single dose to domestic hens (Gallus gallus domesticus) which have been protected from acute cholinergic effects, when appropriate. The animals are observed for at least 21 days for delayed neurotoxicity, with redosing and observation for another 21 days if no effects or equivocal responses are seen. The animals are observed daily for behavioral abnormalities, locomotor ataxia and paralysis. Histopathological examination of selected neural tissues is undertaken on all animals surviving the initial cholinergic phases.

(d) *Test procedures*—(1) *Animal selection.* The adult domestic laying hen, aged 8 to 14 months, is recommended. Standard size breeds and strains should be employed.

(2) *Number of animals.* A sufficient number of hens should be utilized so that at least six survive the observation period.

(3) *Control groups*—(i) *General.* Appropriate control groups should be used. These should include a positive control group of at least two hens treated with a known delayed neurotoxicant and a concurrent control group of at least six hens treated in a manner identical to the treated group, except that administration of the test substance and any protective agents is omitted.

(ii) *Reference substances.* A substance which is known to produce acute delayed neurotoxicity should be used as a positive control. Examples of such substances are triorthocr phosphate (TOCP) and leptophos.

(4) *Housing and feeding conditi* Cages or enclosures which are l enough to permit free mobility of hens and easy observation of should be used. Where the lightin artificial, the sequence should be hours light, 12 hours dark. Appro ate diets should be administered well as an unlimited supply of dr ing water.

(5) *Dose level.* The selected level of the test substance should be less than the unprotected dose. Atropine or another noninter ing protective agent may be use prevent death due to acute choline effects. Doses of test substan higher than 5000 mg/kg of b weight need not be tested.

(6) *Dose selection.* A prelimin LD$_{50}$ test using an appropriate num of animals, dosages and dose gro as recommended in § 798.1175, sh be performed in unprotected hen establish the dose level to be use this test. Healthy young adult h free from interfering viral diseases medication and without abnormal of gait should be acclimatized to laboratory conditions for at lea days prior to randomization and signment to treatment and con groups.

(7) *Route of administration.* Do with the test substance should nor ly be by the oral route using gav gelatine capsules, or a compar method.

(8) *Study conduct*—(i) *General.* test or control substance should be ministered and observations begun hens should be carefully observe least once daily for a period of at l 21 days and signs of toxicity recor including the time of onset, de and duration. Observations should clude, but not be limited to, behavi abnormality, locomotor ataxia and ralysis. At least twice a week the should be taken outside the cages subjected to a period of forced m activity, such as ladder climbing order to enhance the observation minimal responses. If neurotoxic sponses are not observed or if equ cal responses are seen, then the should be administered again and

animals observed for an additional 21 days. The hens should be weighed weekly. Any moribund hens should be removed and sacrificed.

(ii) *Pathology*—(A) *Gross necropsy.* In the presence of clinical signs of delayed neurotoxicity useful information may be provided by gross necropsy.

(B) *Histopathology.* All animals should be subjected to microscopic examination. Tissues should be fixed *in situ*, preferably using perfusion techniques. Sections should include medulla oblongata, spinal cord and peripheral nerves. The spinal cord sections should be taken from the upper cervical bulb, the midthoracic and the lumbo-sacral regions. Section of the proximal region of the tibial nerve and its branches should be taken. Sections should be stained with appropriate myelin and axon-specific stains.

(e) *Data reporting and evaluation*—(1) *Test report.* In addition to the reporting requirements specified under 40 CFR Part 792, Subpart J the final test report must include the following information:

(i) Toxic response data by group with a description of clinical manifestations of nervous system damage; where a grading system is used the criteria should be defined.

(ii) For each animal, time of death during the study or whether it survived to termination.

(iii) The day of observation of each abnormal sign and its subsequent course.

(iv) Body weight data.

(v) Necropsy findings for each animal, when performed.

(vi) A detailed description of all histopathological findings.

(vii) Statistical treatment of results, where appropriate.

(2) *Treatment of results.* Data may be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions or effects, the types of lesions or effects and the percentage of animals displaying each type of lesion or effect.

(3) *Evaluation of results.* The findings of an acute delayed neurotoxicity study should be evaluated in terms of the incidence and severity of neurotoxic effects and of any other observed effects and histopathological findings in the treated and control groups.

(f) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Ankier, S.I. "New hot plate tests to quantify antinociceptic and narcotic antagonist activities," European Journal of Pharmacology *27:* 1-4 (1974).

(2) Coughenour, L.L. McLean, J.R., and Parker R.B. "A new device for the rapid measurement of impaired motor function in mice," Pharmacology, Biochemistry and Behavior *6:* 351-353 (1977).

(3) D'Amour, F.E. Smith, D.L. "A method for determining loss of pain sensation," *Journal of Pharmacology and Experimental Therapeutics, 72:* 74-79 (1941).

(4) Deuel, R.K." Determining sensory deficits in animals," *Methods in Psychobiology Ed.* R.D. Myers (New York: Academic Press, 1977) pp. 99-125.

(5) Edwards, P.M., Parker, V.H. "A simple sensitive and objective method for early assessment of acrylamide neuropathy in rats," *Toxicology and Applied Pharmacology 40:* 589-591 (1977).

(6) Evans, W.O. "A new technique for the investigation of some analgesic drugs on reflexive behavior in the rat," *Psychopharmacologia, 2:* 318-325 (1961).

(7) Irwin, S. "Comprehensive observational assessment: Ia. A systematic quantitative procedure for assessing the behavioral and physiologic state of the mouse," *Psychopharmacologia, 13:* 222-257 (1968).

(8) Marshall, J.F., Turner, B.H., Teitlbaum, P. "Sensory neglect produced by lateral hypothalamic damage," *Science, 174:* 523-525 (1971).

(9) Meyer, O.A, Tilson, H.A., Byrd, W.C., Riley, M.T. "A method for the routine assessment of fore- and hind-limb grip strength of rats and mice," Neurobehavioral Toxicology *1:* 233-236 (1979).

## § 798.6560 Subchronic delayed neuro-toxicity of organophosphorus substances.

(a) *Purpose.* In the assessment and evaluation of the toxic characteristics of organophosphorus substances the determination of subchronic delayed neurotoxicity may be carried out, usually after initial information on delayed neurotoxicity has been obtained by acute testing or by the demonstration of inhibition and aging of neurotoxic esterase in hen neural tissue. The subchronic delayed neurotoxicity test provides information on possible health hazards likely to arise from repeated exposures over a limited period of time. It will provide information on dose response and can provide an estimate of a non-effect level which can be of use for establishing safety criteria for exposure.

(b) *Definitions.* Subchronic delayed neurotoxicity is a prolonged, delayed-onset locomoter ataxia resulting from repeated daily administration of the test substance.

(c) *Principle of the test method.* Multiple dose levels of the test substance are administered orally to domestic hens (*Gallus gallus domesticus*) for 90 days. The animals are observed at least daily for behavioral abnormalities, locomotor ataxia and paralysis. Histopathological examination of selected neural tissues is undertaken at the termination of the test period.

(d) *Test procedures*—(1) *Animal selection.* The adult domestic laying hen, aged 8 to 14 months, is recommended. Standard size breeds and strains should be employed.

(2) *Number of animals.* Ten hens should be used for each treatment and control group.

(3) *Control group*—(i) *General.* A concurrent control group should be used. This group should be treated in a manner identical to the treated group, except that administration of the test substance is omitted.

(ii) *Reference substances.* If a positive control is used, a substance which is known to produce delayed neurotoxicity should be employed. Examples of such substances are triorthocresyl phosphate (TOCP) and leptophos.

(4) *Housing and feeding conditions.* Cages or enclosures which are large enough to permit free mobility of the hens and easy observation of gait should be used. Where the lighting is artificial, the sequence should be 12 hours light, 12 hours dark. Appropriate diets should be administered
well as an unlimited supply of drir
ing water.

(5) *Dose levels.* At least three do
levels should be used in addition
the control group(s). The highest do
level should result in toxic effec
preferably delayed neurotoxicity, b
not produce an incidence of fatalit
which would prevent a meaningf
evaluation. The lowest dose lev
should not produce any evidence
toxicity.

(6) *Route of administration.* Or
dosing each day for at least 5 days p
week should be carried out, preferab
by gavage or administration of gel
tine capsules.

(7) *Study conduct*—(i) *Gener*
Healthy young adult hens free fro
interfering viral diseases and medic
tion and without abnormalities of ga
should be acclimatized to the laborat
ry conditions for at least 5 days prio
to randomization and assignment
treatment and control groups. Th
test or control substance should be a
ministered and observations begun. A
hens should be carefully observed
least once daily throughout the te
period. Signs of toxicity should be r
corded, including the time of onse
degree and duration. Observation
should include, but not be limited t
behavioral abnormality, locomoto
ataxia and paralysis. At least once
week the hens should be taken outsid
the cages and subjected to a period o
forced motor activity, such as ladde
climbing, in order to enhance the observation of minimal responses. Th
hens should be weighed weekly. An
moribund hens should be removed an
sacrificed.

(ii) *Pathology*—(A) *Gross necrops*
In the presence of clinical signs of delayed neurotoxicity useful informatio
may be provided by gross necropsy.

(B) *Histopathology.* Tissues from al
animals should be fixed *in situ,* using
perfusion techniques. Sections should
include medulla oblongata, spinal cor
and peripheral nerves. The spinal cor
sections should be taken from th
upper cervical bulb, the mid-thoraci
and lumbosacral regions. Sections o
the proximal region of the tibial nerv
and its branches and of the sciati
nerve should be taken. Sections should

be stained with appropriate myelin and axon-specific stains. Microscopic examination should be carried out on all hens in the control and high-dose groups. Microscopic examination should also be carried out on hens in the low and intermediate dose groups when there is evidence of effects in the high-dose group.

(e) *Data reporting and evaluation*—(1) *Test report.* In addition to the reporting requirements specified under 40 CFR Part 792, Subpart J the final test report must include the following information:

(i) Toxic response data by group with a description of clinical manifestations of nervous system damage; where a grading system is used the criteria should be defined.

(ii) For each animal, time of death during the study or whether it survived to termination.

(iii) The day of observation of each abnormal sign and its subsequent course.

(iv) Body weight data.

(v) Necropsy findings for each animal, when performed.

(vi) A detailed description of all histopathological findings.

(vii) Statistical treatment of results, where appropriate.

(2) *Treatment of results.* (i) Data may be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions or effects, the types of lesions or effects and the percentage of animals displaying each type of lesion or effect.

(ii) All observed results should be evaluated by an appropriate statistical method. Any generally accepted statistical method may be used; the statistical methods should be selected during the design of the study.

(3) *Evaluation of results.* The findings of a subchronic delayed neurotoxicity study should be evaluated in conjunction with the findings of preceding studies and considered in terms of the incidence and severity of observed neurotoxic effects and any other observed effects and histopathological findings in the treated and control groups. A properly conducted subchronic test should provide a satisfactory estimation of a no-effect level based on lack of clinical signs and histopathological changes.

(f) *References.* For additional background information on this test guideline the following references should be consulted:

(1) Abou-Donia, M.B. "Organophosphorus ester-induced delayed neurotoxicity" Annual Review of Pharmacology and Toxicology, 21:511-548 (1981).

(2) Abou-Donia, M.B., Pressing, S.H. "Delayed neurotoxicity from continuous low-dose oral administration of leptophos to hens." *Toxicology and Applied Pharmacology*, 38:595-608 (1976).

(3) Baron, R.L. (ed). "Pesticide Induced Delayed Neurotoxicity," Proceedings of a Conference, February 19-20, 1976, Washington, DC. U.S. Environmental Protection Agency. EPA Report No. 600/1-76-025, Washington, DC (1976).

(4) Cavanaugh, J.B. "Peripheral neuropathy caused by chemical agents" *Critical Reviews of Toxicity*, 2:365-417 CRC Press, Inc. (1973).

(5) Johannsen, F.R., Wright, P.L., Gordon, D.E., Levinskas, G.L., Radue, R.W., Graham, P.R. "Evaluation of delayed neurotoxicity and dose-response relationship of phosphate esters in the adult hen," *Toxicology and Applied Pharmacology*, 41:291-304 (1977).

(6) Johnson, M.K. "Organophosphorus esters causing delayed neurotoxic effects: mechanism of action and structure/activity studies," *Archives of Toxicology*, 34:259-288 (1975).

## § 798.6850 Peripheral nerve function.

(a) *Purpose.* The techniques in this guideline are designed to develop data on neurophysiological changes in the nervous system for chemical substances and mixtures subject to such testing under the Toxic Substances Control Act. The data will characterize the neurophysiological changes produced by substances known to be peripheral neurotoxicants and determine dose-effect. The EPA will use these data to assess the risk of neurotoxic effects these chemicals may present to human health.

(b) *Definitions.* (1) Neurotoxicity or a neurotoxic effect is an adverse change in the structure or function of the nervous system following exposure to a chemical agent.

(2) Conduction velocity is the speed at which the compound nerve action potential traverses a nerve.

(3) Amplitude is the voltage excursion recorded during the process of recording the compound nerve action potential. It is an indirect measure of the number of axons firing.

(c) *Principle of the test method.* The test substance is administered to several groups of experimental animals, one dose being used per group. The peripheral nerve conduction velocity and amplitude are assessed using electrophysiological techniques. A dose-effect function is determined.

(d) *Test procedure*—(1) *Animal selection*—(i) *Species and strain.* Testing should be performed on a laboratory rodent unless such factors as the comparative metabolism of the chemical or species sensitivity to the toxic effects of the test substance, as evidenced by the results of other studies, dictate otherwise. All animals should have been laboratory-reared to ensure consistency of diet and environmental conditions across groups and should be of the same strain and from the same supplier. If this is not possible, groups shall be balanced to ensure that differences are not systematically related to treatment.

(ii) *Age and weight.* Young adult animals (at least 60 days for rats) must be used. Age (± 15 days for rats) must not vary across groups. Weights should be within ± 10 percent of the mean.

(iii) *Sex.* Either (or both) sex(es) may be used. Sex must not vary across groups.

(2) *Number of animals.* Sufficient numbers of animals shall be used to detect a 10 percent change from normal conduction velocity at the 5 percent level with 90 percent power. Generally, 20 animals/group will satisfy this requirement.

(3) *Control groups.* (i) A concurrent control group is required. This group must be an untreated group, or, if a vehicle is used in administering the test substance, a vehicle control group.

If the toxic properties of the vel
are not known or cannot be n
available, both untreated and vel
control groups are required.

(ii) A satellite group may be tre
with the high dose level for 90
and observed for reversibility, per
ence, or delayed occurrence of toxi
fects for a post-treatment period of
propriate length, normally not
than 28 days.

(4) *Dose levels and dose selection*
least 3 doses, equally spaced on a
scale (e.g., ½ log units) over a rang
at least 1 log unit shall be used in
dition to a zero dose or vehicle adr
istration. The data should be su
cient to produce a dose-effect curve

(i) The highest dose shall prod
(A) clear effects on nerve conduct
velocity and/or amplitude or (B)
threatening toxicity.

(ii) The data from the lower do
must show either (A) graded dose
pendent effects at two dose levels
(B) no effects at two dose levels,
spectively.

(5) *Duration of testing.* The ex
sure duration will be specified in
test rule. This will generally be
days of exposure.

(6) *Route of administration.* T
test substance shall be administe
by a route specified in the test r
This will usually be the route m
closely approximating the route
human exposure. The exposure pro
col shall conform to that outlined
the appropriate acute or subchro
toxicity guideline.

(7) *Combined protocol.* The test
scribed herein may be combined w
any other toxicity study, as long
none of the requirements of either
violated by the combination.

(8) *Study conduct*—(i) *Choice*
*nerve(s).* The nerve conduction velo
ty test must separately assess
properties of both sensory and mo
nerve axons. Either a hind limb (e
tibial) or tail (e.g., ventral caud
nerve must be chosen. Response a
plitude may be measured in a mix
nerve.

(ii) *Preparation.* (A) *In vivo* testi
of anesthetized animals is required.
barbiturate anesthetic is appropria
Care should be taken to ensure th
all animals are administered an equ

alent dosage and that the dosage is not excessive. If dissection is used, extreme caution must be observed to avoid damage to either the nerve or the immediate vascular supply.

(B) Both core and nerve temperature must be monitored and kept constant (±0.5 °C) during the study. Monitoring of skin temperature is adequate if it can be demonstrated that the skin temperature reflects the nerve temperature in the preparation under use. Skin temperature should be monitored with a needle thermistor at a constant site, the midpoint of the nerve segment to be tested.

(C) *Electrodes—(1) Choice of Electrodes.* Electrodes stimulation and recording may be made of any conventional electrode material, such as stainless steel, although electrodes for non-polarizing materials are preferable. If surface electrodes are used, care must be taken to ensure that good electrical contact is achieved between the electrode and the tissue surface. Following each application, any electrode must be throughly cleaned.

(*2*) *Electrode placement.* Electrode placement must be constant with respect to anatomical landmarks across animals (e.g., a fixed number of mm from the base of the tail). Distances between electrodes used to calculate conduction velocity must be measurable to ±0.5 mm. The recording electrodes should be as far from the stimulating electrodes as possible. A 40 mm separation is adequate in the caudal tail nerve of the rat.

(*3*) *Recording conditions.* The animal should be grounded at about the midpoint between the nearest stimulating and recording electrodes. The recording conditions must be such that the stimulus artifact has returned to baseline before any neural response is recorded which is used in the analysis, under condition of maximal band width of the preamplifier.

(D) The electrical stimulator must be isolated from ground. Biphasic or balanced pair stimuli to reduce polarization effects are acceptable. A constant current stimulator is preferred (and required for polarizable electrodes) and should operate from about 10 uA to about 10 mA. If a constant voltage stimulator is used, it should operate to 250V. All equipment shall be calibrated with respect to time, voltage, and temperature.

(E) The recording environment should be enclosed in a Faraday cage unless electromagnetic field pick-up can be shown to be more than 1.5 times the amplifier baseline noise, under recording conditions. The recording output should be amplified sufficiently to render the compound action potential easily measurable with an oscilloscope. The amplifier should pass signals between 2.0 Hz and 4 kHz without more than a 3dB decrement. The preamplifier must be capacitatively coupled or, if direct coupled to the first stages, must be able to tolerate any DC potentials which the electrode-preparation interface produces, and to operate without significant current leakage through the recording electrodes.

(F) A hard copy must be available for all waveforms or averaged waveforms from which measurements are derived, and for all control recording required by this standard. Hard copies must include a time and voltage calibration signal.

(iii) *Procedure—*(A) *General.* Stimulation should occur at inter-stimulus interval significantly below the relative refractory period for the nerve under study. Stimulus intensity should be increased gradually until the response amplitude no longer increases. At this point the "maximal" stimulus current is determined. An intensity 25-50 percent (a fixed value in a given study) above the maximal intensity so determined should be used for determining response peak latency and response amplitude. Response peak latency may be read off the oscilloscope following single sweeps or determined by an average of a fixed number of responses. The baseline-to-peak height technique (Daube, 1980) is acceptable for determination of the nerve compound action potential amplitude, but in this case, at least 16 responses must be averaged.

(B) *Motor nerve.* Motor conduction velocity may be measured from a mixed nerve by recording the muscle action potential which follows the compound action potential of the nerve. The stimulus intensity is adjusted so that the amplitude of the muscle action potential is supramaximal. Measurement of the latency from stimulation to the onset of the compound muscle action potential gives a measure of the conduction time of the motor nerve fibers. To calculate the conduction velocity, the nerve must be stimulated sequentially in two places each with the same cathode-anode distance, and with the cathode located toward the recording electrode. The cathode to cathode distance between the two sets of stimulating electrodes is divided by the difference between the two latencies of muscle action potential in order to obtain conduction velocity. Placement of electrodes shall be described-site of nerve stimulation may differ from point of entry through skin.

(C) *Sensory nerve.* The somatosensory evoked potential may be used to determine the sensory nerve conduction velocity in a mixed nerve. The cathode is placed proximally at the two stimulation locations with the same cathode-anode distances. The recording electrodes are placed on the skull. The conduction velocity is calculated by dividing the distance between the two stimulating cathodes by the difference between the two latencies of the largest primary peak of the somatosensory evoked potential. Between 64 and 128 responses should be averaged. The stimulation frequency should be about 0.5 Hz. Stimulus intensity should be the same as that used for determining the motor conduction velocity. Should the peak of the somatosensory response be so broad that it cannot be replicated with an accuracy of less than 5 percent of the latency difference observed, then a point on the rising phase of the potential should be chosen, e.g. at a voltage 50 percent of the peak voltage. Alternatively, the sensory nerve conduction velocity can be obtained from a purely sensory nerve or from stimulation of the dorsal rootlets of a mixed nerve, using two recording electrode pairs.

(e) *Data collection, reporting and evaluation.* In addition to information meeting the requirements stated under 40 CFR Part 792, Subpart J, the following specific information should be reported:

(1) *Description of test system and test methods.* (i) Positive control data from the laboratory performing the test which demonstrate the sensitivity of the procedure being used.

(ii) Hard copies of waveforms from which measurements were made as well as control recordings.

(iii) Voltage and time calibration referable to the standards of the Bureau of Standards or to other standards of accuracy sufficient for the measurements used.

(iv) Data demonstrating that nerve temperature was maintained constant throughout the recording period.

(2) *Results.* The following information must be arranged by test group (dose level):

(i) In tabular form, data must be provided showing for each animal:

(A) Its identification number.

(B) Body weight, nerve conduction velocity, and amplitude.

(ii) Group summary data should also be reported.

(3) *Evaluation of data.* An evaluation of the test results (including their statistical analysis) must be made and supplied. This submission must include dose-effect curves for conduction velocity and amplitude and a description of statistical methods. Deviation from conventional parametric techniques must be justified.

(f) *References.* For additional background information on this test guidline the following references should be consulted:

(1) Aminoff, M.J. (Ed). *Electrodiagnosis in Clinical Neurology.* (New York: Churchill Livingstone, 1980).

(2) Daube, J. "Nerve Conduction Studies," *Electrodiagnosis in Clinical Neurology.* Ed. M.J. Aminoff (New York: Churchill Livingstone, 1980). pp. 229–264.

(3) Glatt, A.F., H.N. Talaat and W.P. Koella "Testing of peripheral nerve function in chronic experiments in rats," *Pharmacology and Therapeutics,* 5:539–534 (1979).

(4) Johnson, E.W. *Practical Electromyography.* (Baltimore: Williams and Wilkins, 1980).

## Subpart H—Special Studies

### § 798.7100 Metabolism.

(a) *Purpose.* (1) Data from studies on the absorption, distribution, excretion and metabolism of a test chemical are desirable to aid in the evaluation of test results from other toxicology studies and in the extrapolation of data from animals to man. Such studies should be done on each chemical of toxicological concern. The concern may be predicated on the level and type of toxicity observed (or anticipated) and by the magnitude of potential human exposure to the chemical. The main purpose of metabolism studies is to produce data which fortify the understanding of the safety of the chemical in consideration of its intended uses and anticipated human exposure. In addition to the general reasons stated above, a metabolism study may be performed for the following purposes:

(i) To determine the amount and rate of absorption of the test chemical at different dose levels.

(ii) To determine the pattern of distribution of the test chemical among tissues, organs and fluid compartments at different dose levels, after single and repeated doses.

(iii) To identify and, to the extent possible, quantify significant metabolites.

(iv) To characterize route(s) and rate(s) of excretion.

(v) To determine any possible bioaccumulation (bioretention) of the test substance and/or metabolites.

(vi) To determine absorption, metabolism, excretion and distribution as a function of single or repeated doses. For certain chemicals, metabolism studies may not adequately define all of these.

(b) *Definitions.* Bioaccumulation (bioretention) is the uptake and, at least temporary, storage of a chemical by an exposed animal. The chemical can be retained in its original form and/or as modified by enzymatic and non-enzymatic reactions in the body.

(c) *Test procedures*—(1) *Animal selection*—(i) *Species.* The preferred species is the rat. If another mammalian species is used, the tester should provide justification/reasoning for its selection. Commonly used laboratory strains should be employed. Preliminary studies may be performed in more than one species to develop information on comparative metabolism. Information derived from preliminary studies may help in the selection of species for subsequent toxicity tests.

(ii) *Age.* Young adult animals should be used. For specific purposes, a comparative study using very young animals may provide information about the effects of age on metabolism.

(iii) *Sex.* (A) Equal numbers of animals of each sex should be used at each dose level.

(B) Females should be nulliparous and nonpregnant.

(iv) *Numbers.* At least eight animals (four females and four males) should be used at each dose level.

(2) *Dose levels and dose selection.* (i) At least two dose levels should be used.

(ii) The low dose should correspond to a no-observable-effect level.

(iii) The upper dose should produce toxic or pharmacologic signs, but not severe effects or a high incidence of mortality which would prevent a meaningful evaluation.

(iv) The determination of absorption, tissue distribution and elimination should be studied as a function of single and repeated doses.

(v) The conclusive identification of a chemical, and its metabolites, requires the use of suitable analytical methods.

(3) *Observation period.* Animals should be kept in individual metabolism cages for 7 days after the radioactive dose or until 90 percent of the administered dose is excreted (whichever occurs first), at which time all of the animals should be killed.

(4) *Administration of the test substance.* (i) The study should be done using the oral route (capsule or gavage). If another route of administration is used, the tester should provide justification/reasoning for its selection. When vehicles are used, attention should be given to the possibility that they may interfere with the kinetics of the test chemical.

(ii) *Labeled test material:* (A) Single dose testing should be performed with an analytically pure grade of the active ingredient, usually in an isotopically labeled form.

(B) Labeled compound may not be required if sufficiently selective and sensitive physical-chemical tests for identifying the compound and its metabolites are used. The label may be radioactive such as $^{14}C$, $^{35}S$, and $^{36}Cl$ or stable such as $^{15}N$ and $^{18}O$. In some cases, more than one label per molecule may be advantageous. Labels should be placed in positions that may be expected to follow the "core" of the molecule or significant portions thereof. If possible, one should avoid placing labels such as $^{14}C$ in positions from which it may be expected to enter the carbon pool of the test animal. Use of readily exchangeable labeling, should be avoided.

(iii) The following four groups of animals should be studied:

(A) Group A animals shall each receive a single intravenous dose of the labeled test substance at the low dose. If it is not possible to dissolve the test substance in physiological saline or water, this group should be omitted.

(B) Group B animals should each receive a single oral dose of the labeled test substance at the low dose.

(C) Group C animals should each receive a series of single daily oral doses of the nonlabeled test substance (by capsule or intubation) over a period of at least 14 days, followed at 24 hours after the last dose by a single oral dose (by capsule or intubation) of the labeled test substance. Each dose should be at the low dose level.

(D) Group D animals should each receive a single oral dose (by capsule or intubation) of the labeled test substance at the high dose level.

(5) *Observation of animals*—(i) *Distribution*. Concentration and quantity of test chemicals in the tissues and organs should be measured at the time of sacrifice.

(ii) *Metabolism*. For determining the extent of biotransformation, urine samples and fecal extracts should be analyzed by suitable techniques. Major metabolites of the chemical should be identified by appropriate methods. It is also important to determine the metabolite pattern of the test chemical after repeated doses.

(iii) *Excretion*. When determining excretion of the test chemical by laboratory animals, the use of individual metabolism cages is recommended for collection of urine and fecal samples. The quantities of test chemical and major metabolites in urine, feces and in expired air should be measured at several time points after exposure (e.g., 4, 8, 12 and 24 hours) and daily thereafter, until approximately 90 percent of the administered dose has been excreted or until 7 days after dosing.

(iv)(A) In the rat, quantities of label in urine, feces and expired air should be measured at appropriate intervals (e.g., 4, 8, 12, and 24 hours, 2, 3, 4, 5, 6, and 7 days) throughout the study for all animals. However if a preliminary study shows no volatile labeled materials are exhaled during the period of zero to 24 hours after dosing such evidence may be submitted in lieu of measuring label in the expired air for this study.

(B) For all animals in groups B, C, and D, the quantity of label in tissues and organs should be measured at sacrifice by suitable methods with particular attention to bone, brain, fat, gonads, heart, kidney, liver, lungs, muscle, spleen, tissues which displayed pathology (in this or prior studies), and residual carcass.

(v) Urine and feces from all groups should be analyzed by suitable methods in order to determine the extent of absorption and biotransformation and to identify the metabolites. An assay method for detection of each major metabolite may be requested by the Agency.

(d) *Data and reporting*—(1) *Treatment of results*. Data should be summarized in tabular form.

(2) *Evaluation of results*. All observed results, quantitative or incidental, should be evaluated by an appropriate statistical method.

(3) *Test report*. In addition to the reporting requirements as specified under 40 CFR Part 792, Subpart J the following specific information should be reported:

(i) Quantity of isotope, together with percent recovery of the administered dose, in feces, urine, and the following tissues and organs of animals in all groups: Bone, brain, fat, gonads, heart, kidney, liver, lungs, blood, muscle, spleen, tissues which displayed pathology (in this or prior studies), and residual carcass.

(ii) Percent absorption. If possible by the oral route in groups B, C, and D.

(iii) A full description of the sensitivity and precision of all procedures used to produce the data.

(iv) Information on the degree (i.e., specific activity for a radiolabel) and site(s) of labeling of the test substance.

(v) Counting efficiency data should be made available to the Agency upon request.

(vi) Species and strain.

(e) *Additional metabolism studies.* Additional, more specific studies may be required to clarify important points. Some areas for possible further study include: Identification of tissue residues; binding by macromolecules in the blood, liver, gonads and other tissues; placental transfer; entrance into breast milk; biotransformation by specific organs, tissues and cell fractions; and absorption by dermal or inhalation routes of exposure. Plasma binding studies may be conducted, usually *in vitro* with plasma. Placental transfer of a chemical substance may be determined by dosing pregnant rodents with chemicals and assaying their fetuses for the chemical. Additional species may be utilized as those employed may differ significantly in metabolic pattern.

[50 FR 39397, Sept. 27, 1985, as amended at 54 FR 21064, May 16, 1989]

## PART 799—IDENTIFICATION OF SPECIFIC CHEMICAL SUBSTANCE AND MIXTURE TESTING REQUIREMENTS

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799.2175 C9 aromatic hydrocarbon fraction.
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799.2475 2-Mercaptobenzothiazole.
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### Subpart C—Testing Consent Orders

799.5000 Testing consent orders.

### Subpart D—Multichemical Test Rules

799.5055 Hazardous waste constituents subject to testing.

AUTHORITY: 15 U.S.C. 2603, 2611, 2625.

SOURCE: 49 FR 39817, Oct. 10, 1984, unless otherwise noted.

## Subpart A—General Provisions

**§ 799.1 Scope and purpose.**

(a) This part identifies the chemical substances, mixtures, and categories of substances and mixtures for which data are to be developed, specifies the persons required to test (manufacturers, including importers, and/or processors), specifies the test substance(s) in each case, prescribes the tests that are required including the test standards, and provides deadlines for the submission of reports and data to EPA.

(b) This part requires manufacturers and/or processors of chemical substances or mixtures ("chemicals") identified in Subpart B to submit letters of intent to test, exemption applications, and study plans in accordance with EPA test rule development and exemption procedures contained in Part 790 of this chapter and any modifications to such procedures contained in this part.

(c) This part requires manufacturers and/or processors of chemicals identified in Subpart B to conduct tests and submit data in accordance with the test standards contained in this part in order to develop data on the health and environmental effects and other characteristics of these chemicals. These data will be used to assess the risk of injury to human health or the environment presented by these chemicals.

### § 799.2 Applicability.

This part is applicable to each person who manufactures or intends to manufacture (including import) and/or to each person who processes or intends to process a chemical substance or mixture identified in Subpart B for testing during the period commencing with the effective date of the specific chemical test rule until the end of the reimbursement period. Each set of testing requirements in Subpart B specifies whether those requirements apply to manufacturers only, to processors only, or to both manufacturers and processors.

### § 799.3 Definitions.

The definitions in section 3 of the Toxic Substances Control Act (TSCA) and the definitions of § 790.3 of this chapter apply to this part.

### § 799.5 Submission of information.

Information (letters, study plans, reports) submitted to EPA under this Part must bear the Code of Federal Regulations (CFR)a section number of the subject chemical test rule (e.g. § 799.4400 for 1,1,1-trichloroethane) and must be addressed to: TSCA Document Processing Center (TS-790), Rm. L-100, Office of Toxic Substances, Environmental Protection Agency, 401 M St., SW., Washington, DC 20460.

[53 FR 12526, Apr. 15, 1988]

### § 799.10 Test standards.

Testing required under Subpart B must be performed using a study plan prepared according to the requirements of Parts 790 and 792 of this chapter unless modified in specific chemical test rules in Subpart B. All raw data, documentation, records, protocols, specimens and reports generated as a result of a study under Subpart B must be developed, reported, and retained in accordance with TSCA Good Laboratory Practice Standards (GLP's) in Part 792 of this chapter. These items must be made available during an inspection or submitted to EPA upon request by EPA or its authorized representative. Laboratories conducting testing for submission to the Agency in response to a test rule promulgated under section 4 of TSCA must adhere to the TSCA GLP's. Sponsors must notify the laboratory that the study is being conducted pursuant to TSCA section 4. Sponsors are also responsible for ensuring that laboratories conducting the test abide by the TSCA GLP standards. In accordance with § 792.12 of this chapter, a certification concerning adherence to the TSCA GLP's must be submitted to EPA.

### § 799.11 Availability of test guidelines.

(a) The TSCA and FIFRA guidelines for the various study plans are available from the National Technical Information Service (NTIS). Address and telephone number: National Technical Information Service, 5285 Port Royal Road, Springfield, VA 22161 (703-487-4650).

(b) The OECD guidelines for the various study plans are available from the following address: OECD Publication and Information Center, 1750 Pennsylvania Ave., NW., Washington, DC 20006 (202-724-1857).

### § 799.12 Test results.

Except as set forth in specific chemical test rules in Subpart B of this part, a positive or negative test result in any of the tests required under Subpart B is defined in the TSCA test guidelines published by NTIS.

### § 799.17 Effects of non-compliance.

Any person who fails or refuses to comply with any aspect of this part or Part 790 is in violation of section 15 of TSCA. EPA will treat violations of Good Laboratory Practice Standards as indicated in § 792.17 of this chapter.

### § 799.19 Chemical imports and exports.

Persons who export or who intend to export substances listed in Subpart B or Subpart C of this part are subject to the requirements of Part 707 of this title.

[51 FR 23718, June 30, 1986]

## Subpart B—Specific Chemical Test Rules

### § 799.500 Anthraquinone.

(a) *Identification of test substance.* (1) 9,10-anthraquinone (CAS No. 84-65-1) (hereinafter "anthraquinone") shall be tested in accordance with this section.

(2) Anthraquinone of at least 99 percent purity shall be used as the test substance.

(b) *Persons required to submit study plans, conduct tests, and submit data.* All persons who manufacture, import or process anthraquinone, other than as an impurity, from July 20, 1987 to the end of the reimbursement period shall submit letters of intent to conduct testing or exemption applications, submit study plans, conduct tests (in accordance with Part 792 of this chapter), and submit data as specified in this section, Subpart A of this part, and Part 790 of this chapter for single-phase rulemaking.

(c) *First tier chemical fate and environmental effects testing*—(1) *Water solubility*—(i) *Required testing.* Water solubility tests shall be conducted with anthraquinone in accordance with the test guideline specified under § 796.1860 of this chapter. The tests shall be conducted at 12 °C and 22 °C for use in tests with cold and warm water species.

(ii) *Reporting requirements.* (A) The water solubility tests shall be completed and the final results submitted to the Agency within 1 year of the effective date of the final rule.

(A) The water solubility tests shall be completed and the final results submitted to EPA within 15 months of the effective date of the final rule.

(B) A progress report shall be submitted 6 months after the effective date of the final rule.

(2) *Fish acute toxicity*—(i) *Required testing.* (A) Fish acute toxicity tests shall be conducted with anthraquinone using chinook salmon, *Oncorhynchus tshawytscha*, or coho salmon, *Oncorhynchus kisutch* (cold water species); bluegill, *Lepomis macrochirus* (warm water species); and rainbow trout, *Salmo gairdneri* (cold water species) in accordance with the test guideline specified under § 797.1400 of this chapter, except for paragraph (c)(4)(i) of § 797.1400.

(B) For the purposes of this section, the following provisions also apply:

(*1*) A minimum of 20 fish each shall be exposed to each of five or more test substance concentrations. The highest concentration shall be less than or equal to the solubility limit of anthraquinone. At least one concentration shall be between 1 part per billion (ppb) and 10 ppb.

(*2*) The total and dissolved (e.g., filtered) concentrations of the test substance shall be measured in each test chamber and the delivery chamber before the test to ascertain whether it is in solution.

(*3*) The test shall be performed under flowthrough conditions.

(ii) *Reporting requirements.* (A) The fish acute toxicity tests shall be completed and the final results submitted to EPA within 15 months of the effective date of the final rule.

(B) A progress report shall be submitted 6 months after the effective date of the final rule.

(3) *Aquatic invertebrate acute toxicity*—(i) *Required testing.* (A) Aquatic invertebrate acute toxicity tests shall be conducted with anthraquinone using *Daphnia magna* or *D. pulex* and oyster, *Crassostrea virginica*, using the test guidelines specified under §§ 797.1300 and 797.1800 of this chapter, except for paragraph (c)(4)(ii) of § 797.1300.

(B) For the purpose of this section as it relates to § 797.1300 of this chapter, the following provisions also apply:

(*1*) A minimum of 20 daphnids per concentration shall be exposed to five or more concentrations of the test substance chosen in a geometric series in which the ratio is between 1.5 and 2.0 (e.g., 2, 4, 8, 16, 32, and 64 milligrams per liter (mg/L)). The highest concentration shall be less than or equal to the solubility limit of anthraquinone. At least one concentration shall be between 1 ppb and 10 ppb. An equal number of daphnids shall be placed in two or more replicates. If solvents, solubilizing agents or emulsifiers have to be used, they shall be commonly used carriers and shall not possess a synergistic or antagonistic effect on the toxicity of the test chemical. The concentration of solvent shall not exceed 0.1 milliliter per liter (ml/l).

(*2*) The test shall be performed under flowthrough conditions.

(*3*) The total and dissolved (e.g, filtered) concentrations of the test substance shall be measured in each test chamber and the delivery chamber before the test to ascertain whether it is in solution.

(*4*) The stability of the stock solution for the duration of the experiment must be analyzed and reported.

(*5*) The pH of the test solution shall be 7.

(C) For the purpose of this section as it relates to § 797.1800 of this chapter the following provisions also apply:

(*1*) The highest test concentration shall be less than or equal to the solubility limit of anthraquinone.

(*2*) At least one test concentration shall be between 1 ppb and 10 ppb.

(*3*) The total and dissolved (e.g., filtered) concentrations of the test substance shall be measured in each test chamber and the delivery chamber before the test to ascertain whether it is in solution.

(ii) *Reporting requirements.* (A) The invertebrate acute toxicity tests shall be completed and the final results submitted to EPA within 15 months of the effective date of the final rule.

(B) A progress report shall be submitted 6 months after the effective date of the final rule.

(4) *Sediment toxicity to benthic invertebrates*—(i) *Required testing.* A sediment toxicity test shall be conducted using one of the following two methods. (A) *Rhepoxynius* partial life cycle toxicity in sediment: A 10-day toxicity test in a static seawater system shall be conducted with the marine amphipod, *Rhepoxynius abronius*, using clean sediments having low, medium, and high organic carbon content spiked with anthraquinone in the concentration range of 0.01 to 1 part per million (ppm), according to the test guideline specified in the American Society for Testing and Materials Special Technical Testing Publication 854 (ASTM STP 854) entitled, "Phoxocephalid Amphipod Bioassay for Marine Sediment Toxicity," by R.C. Swartz, W.A. DeBen, J.K.P. Jones, J.O. Lamberson, and F.A. Cole and published in *Aquatic Toxicology and Hazard Assessment: Seventh Symposium*, ASTM STP 854, pp. 284–307, R.D. Caldwell, R. Purdy, and R.C. Bahner, Eds., 1985, which is incorporated by reference. (B) *Chironomus* partial life cycle toxicity in sediment: A 14-day toxicity test in a flowthrough system shall be conducted with the freshwater midge, *Chironomus tentans*, using clean, natural sediments having low, medium, and high organic carbon content spiked with anthraquinone in the concentration range of 0.01 to 1 ppm, according to the test guideline specified in the American Society for Testing and Materials Special Technical Testing Publication 854 (ASTM STP 854) entitled, "Aquatic Safety Assessments of Chemicals Sorbed to Sediments," by W.J. Adams, R.A. Kimerle, and R.G. Mosher and published in *Aquatic Toxicology and Hazard Assessment: Seventh Symposium*, ASTM STP 854, pp. 429–452, R.D. Caldwell, R. Purdy, and R.C. Bakner, Eds., 1985, which is incorporated by reference. The ASTM STP 854 is available for inspection at the Office of the Federal Register, Rm. 8401, 1100 L St., NW., Washington, D.C. This incorporation by reference was approved by the Director of the Office of the Federal Register in accordance with 5 U.S.C. 552(a) and 1 CFR Part 51. This material is incorporated as it exists on the effective date of this rule, and a notice of any change in this material will be published in the Federal Register.

Copies of the incorporated material may be obtained from the TSCA Public Docket Office (TS-793), Rm. NE-G004, Office of Toxic Substances, Environmental Protection Agency, 401 M St., SW., Washington, DC 20460, or from the American Society for Testing and Materials (ASTM), 1916 Race Street, Philadelphia, PA 19103.

(ii) *Reporting requirements.* (A) The sediment toxicity test shall be completed and the final results submitted to the Agency within 2 years of the effective date of the final rule.

(B) Progress reports shall be submitted at 6-month intervals beginning 6 months after the effective date of the final rule.

(5) *Bioconcentration*—(i) *Required testing.* (A) A bioconcentration test shall be conducted with anthraquinone using oyster, *Crassostrea virginica*, in accordance with the test guideline specified under § 797.1830 of this chapter, except for paragraphs (c)(4)(ii) and (vi)(A) of § 797.1830.

(B) For the purpose of this section the following provisions also apply:

(*1*) At least two concentrations shall be tested which are at least a factor of 10 apart to assess the propensity of the substance to bioconcentrate. The concentrations selected should not stress or adversely affect the oysters and should be less than one-tenth the EC50 determined in either the range-finding or 96-hour definitive test under § 797.1800 of this chapter. The test concentrations shall be less than the solubility limit of the test substance in water and shall be close to 1 ppb to 10 ppb. The limiting factor of how low one can test is based on the detection limits of the analytical methods. The concentration of the test substance in the test solution should be at least 10 times greater than the detection limit in water.

(*2*) The test shall not be started until the test substance delivery system has been observed to be functioning properly and the test substance concentrations have equilibrated (i.e, the concentration does not vary more than 20 percent). Analyses of two sets of test solution samples taken prior to test initiation should document this equilibrium. At initiation (time 0), the total and dissolved (e.g. filtered) concentrations of test substance shall be measured in the delivery chamber and each test chamber prior to the addition of oysters to the test chambers to ascertain whether it is in solution.

(ii) *Reporting requirements.* (A) The bioconcentration test shall be completed and the final results submitted to the Agency within 18 months of the effective date of the final rule.

(B) Progress reports shall be submitted at 6-month intervals beginning 6 months after the effective date of the final rule.

(d) *Second-tier chemical fate and environmental effects testing.* The following second-tier tests shall be conducted if EPA determines that the total annual volume of anthraquinone manufactured and imported in the United States during a single calendar year exceeds 3 million pounds, and the acute toxicity testing triggers described in this paragraph are met. EPA will monitor the production and importation volume of anthraquinone by the requirement under § 704.30 of this chapter that manufacturers and importers of anthraquinone submit section 8(a) reports to the Agency. EPA will publish notification in the FEDERAL REGISTER or notify the test sponsors by certified letter if the manufacture/importation volume trigger and an acute toxicity trigger are met.

(1) *Biodegradability in activated sludge systems*— (i) *Required testing.* (A) Biodegradability tests in activated sludge systems shall be conducted with anthraquinone in accordance with the test method entitled "Inherent biodegradability: Modified SCAS (semi-continuous activated sludge) test for chemical substances that are water insoluble or water insoluble and volatile" as specified under § 795.45 of this chapter except for paragraphs (b)(2)(i)(E), (F) and (iii)(C) of § 795.45, if EPA determines that the production/importation volume of anthraquinone in the United States during a single calendar year exceeds 3 million pounds, and any of the following conditions is met:

(*1*) The LC50 of the most sensitive fish or the EC50 of the daphnid or oyster, as determined by the acute toxicity tests conducted in accordance with paragraph (c) (2) or (3) of this section, respectively, is less than 100 times the predicted environmental concentration (PEC) in water, i.e., less than 500 ppb;

(*2*) The LC50 of *Rhepoxynius* or *Chironomus*, as determined by the sediment toxicity test conducted in accordance with paragraph (c)(4) of this section, is less than 100 times the PEC in sediment, i.e., less than 10 ppm; or

(*3*) The oyster bioconcentration factor, as determined by the oyster bioconcentration test conducted in accordance with paragraph (c)(5) of this section, is greater than 3,000.

(B) For the purpose of this section the following provisions also apply:

(*1*) A stock solution of $C^{14}$-labeled anthraquinone shall be prepared at a concentration of 2 mg/L which gives a test substance concentration of 0.1 mg/L anthraquinone at the start of each aeration cycle if no biodegradation is occurring.

(*2*) If anthraquinone is insoluble in water at 2 mg/L, it may be necessary to use ultrasound dispersion to obtain a uniform stable suspension. Alternatively, $C^{14}$-labeled anthraquinone may be added directly to the aeration units to give a concentration of 0.1 mg/L anthraquinone at the start of each aeration cycle.

(*3*) One hundred ml of settled sewage are added to the control units, and 95 ml of settled sewage plus 5 ml of the $C^{14}$-labeled anthraquinone stock solution or suspension (2 mg anthraquinone/l) are added to the test units. If test substance is added directly to aeration units, 100 ml of settled sewage are added, as in the control units.

(ii) *Reporting requirements.* (A) The biodegradability tests in activated sludge systems shall be completed and the final results submitted to the Agency within 1 year of the date of EPA's notification of the test sponsor by certified letter or FEDERAL REGISTER notice announcing that the total annual volume of anthraquinone manufactured and imported in the United States during a single calendar year exceeds 3 million pounds and that one or more of the triggers described in paragraph (d)(1)(i) of this section has been met.

(B) A progress report shall be submitted 6 months after EPA's notification of the test sponsor by certified letter or the publication of the FEDERAL REGISTER notice announcing that testing is necessary.

(2) *Biodegradation rate*—(i) *Required testing.* Biodegradation rate tests shall be conducted with anthraquinone at concentrations at or below the water solubility as determined under the testing specified in paragraph (c)(1)(i) of this section, and close to the predicted environmental concentration in sediment, i.e., 0.1 ppm, in accordance with the test guideline described in the article by A.W. Bourquin et al. entitled "An Artificial Microbial Ecosystem for Determining Effects and Fate of Toxicants in a Salt-Marsh Environment," if EPA determines that the production/importation volume of anthraquinone in the United States during a single calendar year exceeds 3 million pounds, and any of the following conditions is met:

(A) The LC50 of the most sensitive fish species or the EC50 for the daphnid or oyster, as determined by the acute toxicity tests conducted in accordance with paragraphs (c) (2) and (3) of this section respectively, is less than 100 times the predicted environmental concentration (PEC) in water, i.e., less than 500 ppb;

(B) The LC50 of *Rhepoxynius* or *Chironomus*, as determined by the sediment toxicity test conducted in accordance with paragraph (c)(4) of this section, is less than 100 times the PEC in sediment, i.e., less than 10 ppm; or

(C) The oyster bioconcentration factor, as determined by the oyster bioconcentration test conducted in accordance with paragraph (c)(5) of this section, is greater than 3,000. The A.W. Bourquin et al. article, entitled "An Artificial Microbial Ecosystem for Determining Effects and Fate of Toxicants in a Salt-Marsh Environment" published in *Developments in Industrial Microbiology*, Vol. 18, Chapter 11, 1977, is incorporated by reference and is available for inspection at the Office of the Federal Register, Rm. 8401, 1100 L St., NW., Washington, DC. This incorporation by reference was approved by the Director of the Office of the Federal Register in accordance with 5 U.S.C. 552(a) and 1 CFR Part 51. This material is incorporated as it exists on the date of approval, and a notice of any change in this material will be published in the FEDERAL REGISTER. Copies of the incorporated material may be obtained from the TSCA Public Docket Office (TS-793), Rm. NE-G004, Office of Toxic Substances, Environmental Protection Agency, 401 M St., SW., Washington, DC 20460, or from the Society for Industrial Microbiology, P.O.Box 12534, Arlington, VA 22209-8534.

(ii) *Reporting requirements.* (A) Biodegradation rate tests shall be completed and the final results submitted to the Agency within 1 year of the date of EPA's notification of the test sponsor by certified letter or a FEDERAL REGISTER notice announcing that the total annual volume of anthraquinone manufactured and imported in the United States during a single calendar year exceeds 3 million pounds and that one or more of the triggers described in paragraph (d)(2)(i) of this section has been met.

(B) A progress report shall be submitted 6 months after EPA's notification of the test sponsor by certified letter or the publication of the FEDERAL REGISTER notice announcing that testing is necessary.

(3) *Fish chronic toxicity*—(i) *Required testing.* (A) Fish chronic toxicity tests shall be conducted with anthraquinone in accordance with the test guideline specified under § 797.1600 of this chapter, except for paragraph (c)(6)(iv) of § 797.1600, if EPA determines that the production/importation volume of anthraquinone in the United States during a single calendar year exceeds 3 million pounds, and if the most sensitive fish species (with the lowest median lethal concentration (LC50)) in the acute toxicity tests conducted in accordance with paragraph (c)(2) of this section has an LC50 less than 100 times the predicted environmental concentration (PEC) in water, i.e., less than 500 ppb.

(B) For the purpose of this section, the following provisions also apply:

(*1*) Prior to the addition of the test substance to the dilution water, it is recommended that the test substance stock solution be analyzed to verify the concentration. After addition of the test substance, the total and dissolved (*e.g.*, filtered) concentrations of the test substance shall be measured at the beginning of the test in each test chamber and delivery chamber to ascertain whether it is in solution. The concentration of test substance shall be measured in one replicate at each test concentration at least once a week thereafter. Replicates should be alternated each week. If a malfunction in the delivery system is discovered, water samples shall be taken immediately from the affected test chambers and analyzed.

(*2*) The highest concentration shall be less than or equal to the solubility limit of anthraquinone.

(*3*) At least one test concentration shall be between 1 ppb and 10 ppb.

(ii) *Reporting requirements.* (A) Fish chronic toxicity tests shall be completed and the final results submitted to the Agency within 2 years of the date of EPA's notification of the test sponsor by certified letter or a FEDERAL REGISTER notice announcing that the total annual volume of anthraquinone manufactured and imported in the United States during a single calendar year exceeds 3 million pounds and that the trigger described in paragraph (d)(3)(i)(A) of this section has been met.

(B) Progress reports shall be submitted at 6-month intervals beginning 6 months after EPA's notification of the test sponsor by certified letter or the publication of the FEDERAL REGISTER notice announcing that testing is necessary.

(4) *Daphnid chronic toxicity*—(i) *Required testing.* (A) Daphnid chronic toxicity test shall be conducted with anthraquinone using *Daphnia magna* or *D. pulex* in accordance with the test guideline specified under § 797.1330 of this chapter, except for paragraph (c)(4)(ii) of § 797.1330, if EPA determines that the total annual volume of anthraquinone manufactured and imported in the United States during a single calendar year exceeds 3 million pounds, and the median effective concentration (EC50) determined in accordance with paragraph (c)(3) of this

section is less than 100 times the PEC in water, i.e., less than 500 ppb.

(B) For the purposes of this section, the following provisions also apply:

(*1*) A minimum of 20 daphnids per concentration shall be exposed to five or more concentrations of the substance chosen in a geometric series in which the ratio is between 1.5 and 2.0, (e.g., 2, 4, 8, 16, 32, 64 mg/L). An equal number of daphnids shall be placed in two or more replicates. The highest concentration shall be less than or equal to the solubility of anthraquinone. At least one concentration shall be between 1 ppb and 10 ppb. Solutions shall be analyzed for chemical concentration prior to use and at designated times during the test.

(*2*) The pH of the test solution shall be 7.

(*3*) The total and dissolved (e.g., filtered) concentrations of test substance shall be measured in each test chamber and the delivery chamber before the test to ascertain whether it is in solution.

(*4*) The test shall be performed under flowthrough conditions;

(*5*) The stability of the stock solution for the duration of the experiment must be analyzed and reported.

(ii) *Reporting requirements.* (A) The daphnid chronic toxicity test shall be completed and the final results submitted to the Agency within 2 years of the date of EPA's notification of the test sponsor by certified letter or a FEDERAL REGISTER notice announcing that the total annual volume of anthraquinone manufactured and imported in the United States during a single calendar year exceeds 3 million pounds and that the trigger described in paragraph (d)(4)(i) of this section has been met.

(B) Progress reports shall be submitted at 6-month intervals beginning 6 months after EPA's notification of the test sponsor by certified letter or the publication of the FEDERAL REGISTER notice announcing that the testing is necessary.

(d) *Effective date.* (1) The effective date of this final rule for anthraquinone is July 20, 1987.

(2) The guidelines and other test methods cited in this section are referenced as they exist on July 20, 1987.

(Information collection requirements have been approved by the Office of Management and Budget under control number 2070-0033)

[52 FR 21028, June 4, 1987, as amended at 53 FR 12526, Apr. 15, 1988; 53 FR 22324, June 15, 1988; 54 FR 27354, June 29, 1989]

EDITORIAL NOTE: At 52 FR 21028, June 4, 1987, § 799.500 was added with two paragraph (d)'s, and at 53 FR 22324, June 15, 1988, the second paragraph (d) was revised. EPA will correctly designate the second paragraph (d) by publishing in the FEDERAL REGISTER a document at a later date.

### § 799.925 Biphenyl.

(a) *Identification of test substance.* (1) Biphenyl (CAS No. 92-52-4) shall be tested in accordance with this rule.

(2) Biphenyl of at least 99 percent purity shall be used as the test substance.

(b) *Persons required to submit study plans, conduct tests and submit data.* All persons who manufacture or process Biphenyl from the effective date of this rule [October 28, 1985] to the end of the reimbursement period shall submit letters of intent to conduct testing or exemption applications, submit study plans, conduct tests and submit data as specified in this section, Subpart A of this part, and Part 790—*Test Rule Development and Exemption Procedures* of this chapter.

(c) *Environmental effects testing*—(1) *Fish early life stage toxicity testing*—(i) *Required testing.* Testing using flow-through systems shall be conducted with rainbow trout to develop data on the chronic toxicity of biphenyl to aquatic vertebrates.

(ii) *Test standard.* The test shall be conducted in accordance with the revised EPA-approved modified study plan submitted to EPA by the Biphenyl Work Group: "Embryo-Larval Toxicity Test with Rainbow Trout, *Salmo gairdneri* Richardson." This revised EPA-approved modified study plan, with modifications approved by EPA on August 7, 1987, and October 16, 1987, is available for inspection in EPA's OPTS Reading Room, Rm. NE G-004, 401 M St. SW., Washington, DC 20460. Copies of this study plan are available to the public in the OPTS reading room.

(iii) *Reporting requirements.* The embryo-larval toxicity test of biphenyl with rainbow trout shall be completed and a final report submitted to the Agency within 72 weeks of the effective date of the final Phase II rule. However, if this study is performed before the flow-through chronic toxicity test with *Daphnia magna* described in paragraph (c)(2) of this section, then the final report for this rainbow trout early-life-stage shall be completed and a final report submitted to the Agency within 42 weeks from the effective date of the final Phase II rule. Progress reports shall be submitted at 6-month intervals beginning 6 months after the effective date of the final Phase II rule.

(2) *Daphnid chronic toxicity testing*—(i) *Required testing.* Testing using flow-through systems shall be conducted with daphnids to develop data on the chronic toxicity of biphenyl to aquatic invertebrates.

(ii) *Test standard.* The test shall be conducted in accordance with the revised EPA-approved modified study plan submitted to EPA by the Biphenyl Work Group: "Flow-Through Chronic Toxicity Test with *Daphnia magna* Straus." This revised EPA-approved modified study plan, with modifications approved by EPA on August 7, 1987, and October 16, 1987, is available for inspection in EPA's OPTS Reading Room, Rm. NE G-004, 401 M St. SW., Washington, DC 20460. Copies of this study plan are available to the public in the OPTS reading room.

(iii) *Reporting requirements.* The flow-through chronic toxicity test of biphenyl with *Daphnia magna* shall be completed and a final report submitted to the Agency within 30 weeks from the effective date of the final Phase II rule. However, if the embryo-larval toxicity test with rainbow trout described in paragraph (c)(1) of this section is performed before this study, then the final report for this chronic *Daphnia magna* study shall be completed and a final report submitted to the Agency within 72 weeks from the effective date of the final Phase II rule. Progress reports shall be submitted at 6-month intervals beginning 6 months after the effective date of the final Phase II rule.

(3) *Oyster acute toxicity testing*—(i) *Required testing.* Testing using systems that control for biphenyl evaporation shall be conducted with oysters to develop data on the acute toxicity of sediment-associated biphenyl to benthic invertebrates.

(ii) *Test standard.* The testing shall be conducted in accordance with the revised EPA-approved modified study plan submitted to EPA by the Biphenyl Work Group: "Oyster Shell Deposition Bioassay and Range-finding Study". This revised EPA-approved modified study plan is available for inspection in EPA's OPTS Reading Room, Rm. NE-G004, 401 M Street, SW., Washington, DC 20460; copies of this study plan are available for distribution to the public in the OPTS Reading Room.

(iii) *Reporting requirements.* The oyster shell deposition and range-finding study with biphenyl shall be completed and a final report submitted to the Agency within 65 weeks from the effective date of the final Phase II rule. Progress reports shall be submitted at 6-month intervals beginning 6 months after the effective date of the final Phase II rule.

(4) *Oyster bioconcentration testing*—(i) *Required testing.* Testing using systems that control for biphenyl evaporation shall be conducted with oysters to develop data on the potential chronic toxicity and bioconcentration of sediment-associated biphenyl to benthic invertebrates.

(ii) *Test standard.* The testing shall be conducted in accordance with the revised EPA-approved modified study plan submitted to EPA by the Biphenyl Work Group: "Flow-Through Oyster Bioconcentration Study". This revised EPA-approved modified study plan is available for inspection in EPA's OPTS Reading Room, Rm. NE-G004, 401 M Street, SW., Washington, DC 20460; copies of this study plan are available for distribution to the public in the OPTS Reading Room.

(iii) *Reporting requirements.* The oyster bioconcentration study shall be completed and a final report submitted to the Agency within 67 weeks from the effective date of the final

Phase II rule. Progress reports shall be submitted at 6-month intervals beginning 6 months after the effective date of the final Phase II rule.

(d) *Chemical fate testing*—(1) *Aerobic biodegradation*—(i) *Required testing*. Testing using systems that control for and quantify biphenyl evaporation that use a ratio of undisturbed sediment to water of 3:1—2:1 and that provide a mass balance of biphenyl distributed in water and sediment, volatilized or degraded to $CO_2$ or other products before and after biodegradation shall be conducted to develop data on the persistence of biphenyl in aerobic sediments.

(ii) *Test standard*. The testing shall be conducted in accordance with the revised EPA-approved modified study plan submitted to EPA by the Biphenyl Work Group: "Aerobic Biodegradation Study." This revised EPA-approved modified study plan, with modifications approved by EPA on October 13, 1987, is available for inspection in EPA's OPTS Reading Room, Rm. NE G-004, 401 M St. SW., Washington, DC 20460. Copies of this study plan are available to the public in the OPTS reading room.

(iii) *Reporting requirements*. The aerobic biodegradation study with biphenyl shall be completed and a final report submitted to the Agency within 52 weeks of the effective date of the final Phase II rule. Progress reports shall be submitted at 6-month intervals beginning 6 months after the effective date of the final Phase II rule.

(2) *Anaerobic biodegradation*—(i) *Required testing*. Testing using systems that control for and quantify biphenyl evaporation that use a ratio of undisturbed sediment to water of 3:1—2:1 and that provide a mass balance of biphenyl distributed in water and sediment, volatilized or degraded to $CO_2$ or other products before and after biodegradation shall be conducted with biphenyl to develop data on the persistence of biphenyl in anaerobic sediments.

(ii) *Test standard*. The testing shall be conducted in accordance with the revised EPA-approved modified study plan submitted to EPA by the Biphenyl Work Group: "Anaerobic Biodegradation Study." This revised EPA-approved modified study plan, with modifications approved by EPA on October 13, 1987, is available for inspection in EPA's OPTS Reading Room, Rm. NE G-004, 401 M St. SW., Washington, DC 20460. Copies of this study plan are available to the public in the OPTS reading room.

(iii) *Reporting requirements*. The anaerobic biodegradation study with biphenyl shall be completed and a final report submitted to EPA within 64 weeks of the effective date of the final Phase II rule. Progress reports shall be submitted at 6-month intervals beginning 6 months after the effective date of the final Phase II rule.

(3) *Partitioning water/sediment study*—(i) *Required testing*. Testing using systems that control for and quantify biphenyl evaporation that use a ratio of undisturbed sediment to water of 1:3 shall be conducted with biphenyl to develop data on the partitioning of biphenyl to water and sediment.

(ii) *Test standard*. The testing shall be conducted in accordance with the revised EPA-approved modified study plan submitted to EPA by the Biphenyl Work Group: "Partitioning Water/Sediment Study." This revised EPA-approved modified study plan, with modifications approved by EPA on October 13, 1987, is available for inspection in EPA's OPTS Reading Room, Rm. NE G-004, 401 M St., SW., Washington, DC 20460. Copies of this study plan are available to the public in the OPTS reading room.

(iii) *Reporting requirements*. The partitioning water/sediment testing shall be completed and a final report submitted to EPA by June 1, 1988, for the river test, and by July 15, 1988, for the lake test. Progress reports shall be submitted to EPA at 6-month intervals beginning 6 months after the effective date of the final Phase II rule.

(e) *Effective date*. The effective date of the final Phase II rule for biphenyl is July 17, 1987.

(Information collection requirements approved by the Office of Management and Budget under control number 2070-0033)

[50 FR 37188, Sept. 12, 1985, as amended at 52 FR 20713, June 3, 1987; 54 FR 27384, June 29, 1989]

**§ 799.940 Bisphenol A.**

(a) *Identification of test substance.* (1) Bisphenol A (CAS Number 80-05-7) (hereinafter"BPA") shall be tested in accordance with this section.

(2) BPA of at least 99 percent purity shall be used as the test substance.

(3) BPA shall be administered as a dust for inhalation with a target mass median aerodynamic diameter of 0.1 to 5 micrometers.

(b) *Persons required to submit study plans, conduct tests, and submit data.* All persons who manufacture or process BPA, other than as an inpurity, November 3, 1986 to the end of the reimbursement period shall submit letters of intent to conduct testing, submit study plans, conduct tests, and submit data or submit exemption applications as specified in this section, Subpart A of this part, and Parts 790 and 792 of this chapter for single-phase rulemaking.

(c) *Health effects testing*—(1) *Required inhalation toxicity testing.* Subchronic toxicity and recovery testing including the satellite test group, shall be conducted with BPA in accordance with the TSCA Health Effects Test Guideline for Inhalation Toxicity in § 798.2450(a), (b), (c) and (e) of this chapter. The following additional testing requirements apply to bisphenol A:

(i) *Test procedures*—(A) *Animal selection*—(*1*) *Species and strain.* A mammalian species shall be used for testing. A variety of rodent species may be used although the rat is the preferred species. Commonly used laboratory strains shall be employed. If another mammalian species is used, the tester shall provide justification/reasoning for its selection.

(*2*) *Age.* Young adult animals shall be used. At the commencement of the study the weight variation of animals shall not exceed ±20 percent of the mean weight for each sex.

(*3*) *Sex.* (*i*) Equal numbers of animals of each sex shall be used at each dose level.

(*ii*) Females shall be nulliparous and nonpregnant.

(*4*) *Numbers.* (*i*) At least 20 animals (10 females and 10 males) shall be used for each test group.

(*ii*) If interim sacrifices are planned, the number of animals shall be increased by the number of animals scheduled to be sacrificed before the completion of the study.

(B) *Control groups.* A concurrent control group is required. This group shall be an untreated or sham-treated control group. Except for treatment with the test substance, animals in the control group shall be handled in a manner identical to the test group animals. Where a vehicle is used to help generate an appropriate concentration of the substance in the atmosphere, a vehicle control group shall be used. If the toxic properties of the vehicle are not known or cannot be made available, both untreated and vehicle control groups are required.

(C) *Satellite group.* A satellite group of 20 animals (10 animals per sex) shall be treated with the high concentration level for 90 days and observed for reversibility, persistence, or delayed occurrence of toxic effects for a posttreatment period of not less than 28 days.

(D) *Dose levels and dose selection.* (*1*) In subchronic toxicity tests, it is desirable to have a dose-response relationship as well as a no-observed-toxic-effect level. Therefore, at least three dose levels with a control and, where appropriate, a vehicle control (corresponding to the concentration of vehicle at the highest exposure level) shall be used. Doses should be spaced appropriately to produce test groups with a range of toxic effects. The data should be sufficient to produce a dose-response curve.

(*2*) The highest concentration should result in toxic effects but not produce an incidence of fatalities which would prevent a meaningful evaluation.

(*3*) The lowest concentration should not produce any evidence of toxicity. Where there is a usable estimation of human exposure, the lowest concentration should exceed this.

(*4*) Ideally, the intermediate dose level(s) should produce minimal observable toxic effects. If more than one intermediate dose level is used, the concentrations should be spaced to produce a gradation of toxic effects.

(*5*) In the low and intermediate groups and in the controls the incidence of fatalities should be low, to permit a meaningful evaluation of the results.

(*6*) In the case of potentially explosive test substances, care should be taken to avoid generating explosive concentrations.

(E) *Exposure conditions.* The animals should be exposed to the test substance ideally for 6 hours per day on a 7 day per week basis, for a period of 90 days. However, based primarily on practical considerations, exposure on a 5-day per week basis for 6 hours per day is the minimum acceptable exposure period.

(F) *Observation period.* (*1*) Duration of observation shall be for atleast 90 days.

(*2*) Animals in a satellite group scheduled for followup observations shall be kept for an additional minimum 28 days without treatment to detect recovery from, or persistence of, toxic effects.

(G) *Inhalation exposure.* (*1*) The animals shall be tested in inhalation equipment designed to sustain a dynamic air flow of 12 to 15 air changes per hour and ensure an adequate oxygen content of 19 percent and an evenly distributed exposure atmosphere. Where a chamber is used, its design should minimize crowding of the testing animals and maximize their exposure to the test substance. This is best accomplished by individual caging. To ensure stability of a chamber atmosphere, the total "volume" of the test animals shall not exceed 5 percent of the volume of the test chamber. Oronasal or head-only exposure may be used if it is desirable to avoid concurrent exposure by the dermal or oral routes.

(*2*) A dynamic inhalation system with a suitable analytical concentration control system shall be used. The rate of air flow shall be adjusted to ensure that conditions throughout the exposure chamber are essentially the same. Maintenance of slight negative pressure inside the chamber will prevent leakage of the test substance into the surrounding areas.

(*3*) The temperature at which the test is performed shall be maintained at 22° C (+2°). Ideally, the relative humidity shall be maintained between 40 to 60 percent.

(H) *Physical measurements.* Measurements or monitoring shall be made of the following:

(*1*) The rate of air flow should be monitored continuously but shall be recorded at least every 30 minutes.

(*2*) The actual concentrations of the test substance shall be measured in the breathing zone. During the exposure period the actual concentrations of the test substance should be held as constant as practicable and monitored continuously and shall be recorded at least at the beginning, at an intermediate time and at the end of the exposure period.

(*3*) During the development of the generating system, particle size analysis shall be performed to establish the stability of aerosol concentrations. During exposure, analysis shall be conducted as often as necessary to determine the consistency of particle size distribution.

(*4*) Temperature and humidity shall be monitored continuously and shall be recorded at least every 30 minutes.

(I) *Food and water during exposure period.* Food shall be withheld during exposure. Water may also be withheld if necessary.

(J) *Observation of animals.* (*1*) Each animal should be handled and its physical condition shall be appraised at least once each day.

(*2*) Additional observations should be made daily with appropriate actions taken to minimize loss of animals to the study (e.g. necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals).

(*3*) Signs of toxicity shall be recorded as they are observed including the time of onset, the degree, and duration.

(*4*) Cage-sided observations should include but not be limited to changes in the skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous sytems, somatomotor activity and behavior pattern.

(*5*) Animals shall be weighed weekly. Food consumption should also be determined weekly if abnormal body weight changes are observed.

(*6*) At the end of the study period all survivors in the nonsatellite treatment groups shall be sacrificed. Moribund animals shall be removed and sacrificed when noticed.

(K) *Clinical examinations.* (*1*) The following examinations shall be made on at least five animals of each sex in each group:

(*i*) Certain hematology determinations shall be carried out at least two times during the test period: At terminal sacrifice at the end of the 90-day test period and at completion of the post-exposure recovery period (satellite group). Hematology determinations which shall be appropriate to this study include: packed cell volume, hemoglobin, erythrocyte count, total leukocyte, red blood cell indices, platelet count, and differential leukocyte count.

(*ii*) Certain clinical biochemistry determinations on blood shall be carried out at least two times: At terminal sacrifice at the end of the 90-day test period and at completion of the post-exposure recovery period (satellite group). Clinical biochemistry test areas which shall be appropriate to this study include: blood urea nitrogen, glutamic pyruvic transaminase activity, glutamic oxaloacetic transaminase activity, alkaline phosphatase activity, glucose, total protein, albumin, and globulins. Other determinations which may be necessary for an adequate toxicological evaluation include: analyses of lipids, hormones, methemoglobin, and cholinesterase activity. Additional clinical biochemistry may be employed, where necessary, to extend the investigation of observed effects.

(*2*) The following examinations shall be made on at least five animals of each sex in each group:

(*i*) Ophthalmological examination, using an ophthalmoscope or equivalent suitable equipment, shall be made prior to exposure to the test substance and at the termination of the study. If changes in the eyes are detected, all animals should be examined.

(*ii*) Urinalysis is not recommended on a routine basis, but only when there is an indication based on expected or observed toxicity.

(L) *Gross pathology.* (*1*) All animals shall be subjected to a full gross necropsy which includes examination of the external surface of the body, all orifices and the cranial, thoracic and abdominal cavities and their contents, and the esophagus, stomach, and upper small intestine.

(*2*) At least the liver, kidneys, brain, and male gonads shall be weighed wet, as soon as possible after dissection to avoid drying.

(*3*) The following organs and tissues, or representative samples thereof, shall be preserved in a suitable medium for possible future histopathological examination: All gross lesions; lungs—which shall be removed intact, weighed, and treated with a suitable fixative to ensure that lung structure is maintained (perfusion with the fixative is considered to be an effective procedure); nasopharyngeal tissues; brain—including sections of medulla/pons cerebellar cortex and cerebral cortex; pituitary; thyroid/parathyroid; thymus; trachea; heart; sternum with bone marrow; salivary glands; liver; spleen; kidneys; adrenals; pancreas; gonads; uterus; accessory genital organs, epididymis, prostate, and, if present, seminal vesicles; aorta; skin; gall bladder (if present); esophagus; stomach; duodenum; jejunum; ileum; cecum; colon; rectum; urinary bladder; representative lymph node; mammary gland; thigh musculature; peripheral nerve; eyes; femur—including articular surface; spinal cord at three levels—cervical, midthoracic, and lumbar; and exorbital lachrymal glands.

(M) *Histopathology.* The following histopathology shall be performed: (*1*) Full histopathology on the respiratory tract including nasal cavity, pharynx, larynx and paranasal sinuses of all animals in the control, high dose, and satellite groups.

(*2*) All gross lesions in all animals.

(*3*) Target organs in all animals.

(*4*) Lungs of animals in the low and intermediate dose groups shall also be subjected to histopathological examination contingent on the histopathological findings of the control, high dose, and satellite groups.

(5) When a satellite group is used, histopathology shall be performed on tissues and organs identified as showing effects in other treated groups.

(ii) [Reserved]

(2) *Reporting requirements.* (i) Subchronic toxicity testing, including the satellite test group, shall be completed and the final study report submitted to the Agency within 17 months from the effective date of this final rule.

(ii) Progress reports shall be suumitted at 6 month intervals, the first of which is due within 6 months of the effective date of this final rule.

(Information collection requirements have been approved by the Office of Management and Budget under control number 2070-0033)

[51 FR 33052, Sept. 18, 1986, as amended at 52 FR 1331, Jan. 13, 1987]

### § 799.1051 Monochlorobenzene.

(a) *Identification of test substance.* (1) Monochlorobenzene (CAS Number 108-90-7) (hereinafter "MCB") shall be tested in accordance with this section.

(2) MCB of at least 99 percent purity shall be used as the test substance.

(3) The test substance shall not contain more than 0.05 percent benzene and 0.05 percent hexachlorobenzene.

(b) *Persons required to submit study plans, conduct tests and submit data.* All persons who manufacture (import) or process monochlorobenzene other than as an impurity after the effective date of this rule (August 21, 1986) to the end of the reimbursement period shall submit letters of intent to conduct testing or exemption applications, submit study plans, conduct tests, and submit data as specified in this section, Subpart A of this part, and Parts 790 and 792 of this chapter for single-phase rulemaking.

(c) *Health effects testing*—(1) *Reproductive and fertility effects*—(i) *Required testing.* (A) A test for reproductive and fertility effects shall be conducted with MCB in accordance with § 798.4700 of this chapter.

(B) The route of administration for the reproductive and fertility effects testing of MCB shall be inhalation.

(C) The test species shall be the Sprague-Dawley Rat.

(ii) *Reporting requirements.* (A) The reproductive and fertility effects test shall be completed and the final results submitted to the Agency within 29 months of the effective date of this rule.

(B) Progress reports shall be submitted to the Agency every 6 months after the effective date of the final rule.

Approved by the Office of Management and Budget under control number 2070-0033)

[51 FR 24666, July 8, 1986]

### § 799.1052 Dichlorobenzenes.

(a) *Identification of test substances.* (1) 1,2,- and 1,4-dichlorobenzenes, CAS Numbers 95-50-1 and 106-46-7 respectively, shall be tested in accordance with this section.

(2) The substances identified in paragraph (a)(1) of this section shall be 99 percent pure and shall be used as the test substances in each of the tests specified.

(3) For health effects testing required under paragraph (e) of this section, both test substances shall not contain more than 0.05 percent benzene and 0.05 percent hexachlorobenzene.

(b) *Persons required to submit study plans, conduct tests, and submit data.* (1) All persons who manufacture or process substances identified in paragraph (a)(1) of this section, other than as an impurity, from May 21, 1986, to the end of the reimbursement period, shall submit letters of intent to test or exemption applications and shall conduct tests, in accordance with Part 792 of this chapter, and submit data as specified in this section, Subpart A of this part and Part 790 of this chapter for two-phase rulemaking.

(2) Persons subject to this section are not subject to the requirements of § 790.50(a) (2), (5), (6) and (b) and § 790.87(a)(1)(ii) of this chapter.

(3) Persons who notify EPA of their intent to conduct tests in compliance with the requirements of this section must submit plans for those tests no later than 30 days before the initiation of each of those tests.

(4) In addition to the requirements of § 790.87(a)(2) and (3) of this chapter, EPA will conditionally approve ex-

emption applications for this rule if EPA has received a letter of intent to conduct the testing from which exemption is sought and EPA has adopted tests standards and schedules in a final Phase II test rule.

(5) For health effects testing required under paragraph (e) of this section, all persons who manufacture (import) or process 1,2- and/or 1,4-dichlorobenzene, other than as an impurity, after the effective date of this rule (August 21, 1986) to the end of the reimbursement period, for each of these chemicals that they manufacture and/or process, shall submit letters of intent to conduct testing or exemption applications, submit study plans, conduct tests, and submit data as specified in this section, Subpart A of this part, and Parts 790 and 792 of this chapter for single-phase rulemaking.

(c) [Reserved]

(d) *Health effects testing*—(1) *Reproductive and fertility effects*—(i) *Required testing.* (A) A test for reproductive and fertility effects shall be conducted with both 1,2- and 1,4-DCBs in accordance with § 798.4700 of this chapter.

(B) The route of administration for the reproductive and fertility effects testing of both 1,2- and 1,4-DCB shall be inhalation.

(C) The test species shall be the Sprague-Dawley rat.

(ii) *Reporting requirements.* (A) Both reproductive and fertility effects tests shall be completed and the final results submitted to the Agency within 29 months of the effective date of this final rule.

(B) Progress reports for both studies shall be submitted to the Agency every 6 months after the effective date of the final rule.

(C) Study plans shall be submitted to the Agency no later than the initiation of each of the tests.

(Approved by the Office of Management and Budget under control number 2070-0033)

[51 FR 11736, Apr. 7, 1986, as amended at 51 FR 24667, July 8, 1986; 52 FR 10378, Apr. 1, 1987; 52 FR 24465, July 1, 1987]

## § 799.1053 Trichlorobenzenes.

(a) *Identification of testing substance.* (1) 1,2,3- and 1,2,4-trichlorobenzenes, CAS Numbers 87-61-6 and 120-82-1 respectively, shall be tested in accordance with this section.

(2) The substances identified in paragraph (a)(1) of this section shall be 99 percent pure and shall be used as the test substances in each of the tests specified.

(3) For health effects testing required under paragraph (e) of this section, the test substance shall not contain more than 0.05 percent benzene and 0.05 percent hexachlorobenzene.

(b) *Persons required to submit study plans, conduct tests, and submit data.* (1) All persons who manufacture or process substances identified in paragraph (a)(1) of this section, other than an impurity, from May 21, 1986, to the end of the reimbursement period, shall submit a letter of intent to test or exemption applications and shall conduct tests, in accordance with Part 792 of this chapter, and submit data as specified in this section, Subpart A of this part and Part 790 of this chapter for two-phase rulemaking.

(2) Persons subject to this section are not subject to the requirements of § 790.50(a) (2), (5), (6) and (b) and § 790.87(a)(1)(ii) of this chapter.

(3) Persons who notify EPA of their intent to conduct tests in compliance with the requirements of this section must submit plans for those tests no later than 30 days before the initiation of each of those tests.

(4) In addition to the requirements of § 790.87(a)(2) and (3) of this chapter, EPA will conditionally approve exemption applications for this rule if EPA has received a letter of intent to conduct the testing from which exemption is sought and EPA has adopted test standards and schedules in a final Phase II test rule.

(5) For health effects testing required under paragraph (e) of this section, all persons who manufacture (import) or process 1,2,4-trichlorobenzene, other than as an impurity, after the effective date of this rule (August 21, 1986) to the end of the reimbursement period shall submit letters of intent to conduct testing or exemption

applications, submit study plans, conduct tests, and submit data as specified in this section, Subpart A of this part, and Parts 790 and 792 of this chapter for single-phase rulemaking.

(c) [Reserved]

(d) *Environmental effects testing.* 1,2,3- and 1,2,4-trichlorobenzenes shall be tested in accordance with this section.

(1) *Marine invertebrate acute toxicity testing*—(i) *Required testing.* Testing using measured concentrations, flow through or static renewal systems, and systems that control for evaporation of the test substance, shall be conducted for 1,2,3- and 1,2,4-trichlorobenzenes. Testing shall be conducted with mysid shrimp (*Mysidopis bahia*) to develop data on the acute toxicity of the above chlorobenzene isomers to marine invertebrates.

(ii) *Test standards.* The marine invertebrate (mysid shrimp, *Mysidopis bahia*) acute toxicity testing for 1,2,3- and 1,2,4-trichlorobenzenes shall be conducted in accordance with § 797.1930 of this chapter.

(iii) *Reporting requirements.* (A) The acute toxicity tests on marine invertebrates shall be completed and the final report submitted to EPA within 1 year of the effective date of the final Phase II test rule.

(B) An interim progress report shall be submitted to the Agency within 6 months after the effective date of the final Phase II rule.

(2) *Marine fish acute toxicity testing*—(i) *Required testing.* Testing using measured concentrations, flow through systems, and systems that control for evaporation of the test substance shall be conducted for 1,2,3-trichlorobenzene. Testing shall be conducted with Silversides (*Menidia menidia*) to develop data on the acute toxicity of 1,2,3-trichlorobenzene to saltwater fish.

(ii) *Test standard.* The marine fish (silverside minnow, *Menidia menidia*) acute toxicity test shall be conducted for 1,2,3-trichlorobenzene in accordance with § 797.1400 of this chapter.

(iii) *Reporting requirements.* (A) The marine fish (silversides minnow, *Menidia menidia*) acute toxicity test shall be completed and the final results submitted within 1 year of the effective date of the Phase II final test rule.

(B) An interim progress report shall be submitted to EPA 6 months after the effective date of the final Phase II rule.

(3) *Freshwater fish acute toxicity testing*—(i) *Required testing.* Testing using measured concentrations, flow through systems, and systems that control evaporation of the test substance shall be conducted for 1,2,3-trichlorobenzene. A 96-hour LC50 test shall be conducted with the fathead minnow (*Pimephales promelas*) to develop data on the acute toxicity of 1,2,3-trichlorobenzene to freshwater fish.

(ii) *Test standard.* The freshwater fish (fathead minnow, *Pimephales promelas*) acute toxicity test shall be conducted for 1,2,3-trichlorobenzene in accordance with § 797.1400 of this chapter.

(iii) *Reporting requirements.* (A) The freshwater fish acute toxicity study shall be completed and the final report submitted to EPA within 1 year of the effective date of the final Phase II test rule.

(B) An interim progress report shall be submitted to EPA 6 months after the effective date of the final Phase II rule.

(4) *Freshwater invertebrate acute toxicity testing*—(i) *Required testing.* Testing using measured concentrations, flow through or static renewal systems, and systems that control for evaporation of the test substance shall be conducted for 1,2,3-trichlorobenzene. A 96-hour EC50 shall be conducted for one species of *Grammarus* to develop data on the acute toxicity of 1,2,3-trichlorobenzene to aquatic freshwater invertebrates.

(ii) *Test standard.* The freshwater invertebrate (Gammarus sp.) acute toxicity test shall be conducted for 1,2,3-trichlorobenzene in accordance with § 795.120 of this chapter.

(iii) *Reporting requirements.* (A) The freshwater invertebrate acute toxicity test shall be completed and the final report submitted to EPA within 1 year of the effective date of the final Phase II rule.

(B) An interim progress report shall be submitted to EPA 6 months after the effective date of the final Phase II rule.

(5) *Mysid shrimp chronic toxicity testing*—(i) *Required testing.* Testing using measured concentrations, flow through or static renewal systems, and systems that control for evaporation of the test substance shall be conducted for 1,2,4-trichlorobenzene. Testing shall be conducted with mysid shrimp (*Mysidopsis bahia*) to develop data on the chronic toxicity of 1,2,3-trichlorobenzene, should the acute LC50 of this chemical to mysid shrimp be determined to be less than 1 ppm.

(ii) *Test standards.* The mysid shrimp (*Mysidopis bahia*) chronic toxicity test shall be conducted for 1,2,4-trichlorobenzene in accordance with § 797.1950 of this chapter. Testing shall also be conducted according to § 797.1950 for 1,2,3-trichlorobenzene should the results of testing required by (d)(1)(ii) of this section yield an acute LC50 for this chemical substance of less than 1 ppm.

(iii) *Reporting requirements.* (A) The mysid shrimp chronic toxicity test for 1,2,4-trichlorobenzene shall be completed and the final report submitted to EPA within 1 year of the effective date of the final Phase II rule. The mysid shrimp chronic toxicity test for 1,2,3-trichlorobenzene, (required if the LC50 is less than 1 ppm), shall be completed and final report submitted to EPA within 15 months of the effective date of the final Phase II rule.

(B) Progress reports shall be submitted to EPA at 6-month intervals, beginning 6 months after of the effective date of the final Phase II rule and until the final report is submitted to EPA.

(e) *Health effects testing*—(1) *Oncogenicity*—(i) *Required testing.* (A) A test for oncogenic effects shall be conducted with 1,2,4-TCB in accordance with § 798.3300 of this chapter.

(B) The route of administration for the oncogenicity testing for 1,2,4-TCB shall be via the animal feed.

(C) Two rodent species shall be used and one shall be the Fischer-344 rat.

(ii) *Reporting requirements.* (A) The oncogenicity test shall be completed and the final results submitted to the Agency within 53 months of the effective date of this final rule.

(B) Progress reports shall be submitted to the Agency every 6 months after the effective date of the final rule.

(f) [Reserved]

(g) *Effective date.* The effective date of the final Phase II rule is August 14, 1987.

(Information collection requirements have been approved by the Office of Management and Budget under control number 2070-0033)

[51 FR 11737, Apr. 7, 1986; 51 FR 18444, May 20, 1986, as amended at 51 FR 24667, July 8, 1986; 52 FR 24465, July 1, 1987]

### § 799.1054 1,2,4,5-Tetrachlorobenzene.

(a) *Identification of test substances.* (1) 1,2,4,5-Tetrachlorobenzene (CAS Number 95-94-3) (hereinafter "1,2,4,5-TCB") shall be tested in accordance with this section.

(2) 1,2,4,5-TCB of at least 97 percent purity shall be used as the test substance.

(3) The test substance shall not contain more than 0.05 percent benzene and 0.05 percent hexachlorobenzene.

(b) *Persons required to submit study plans, conduct tests and submit data.* All persons who manufacture (import) or process 1,2,4,5-tetrachlorobenzene, other than as an impurity, after the effective date of this rule (August 21, 1986) to the end of the reimbursement period shall submit letters of intent to conduct testing or exemption applications, submit study plans, conduct tests, and submit data as specified in this section, Subpart A of this part, and Parts 790 and 792 of this chapter for single-phase rulemaking.

(c) *Health effects testing*—(1) *Reproduction and fertility*—(i) *Required testing.* (A) A test for reproduction and fertility effect shall be conducted with 1,2,4,5-TCB in accordance with § 798.4700 of this chapter.

(B) The route of administration for the reproduction and fertility testing for 1,2,4,5-TCB shall be dietary.

(C) A rodent test species shall be used and shall be the Sprague-Dawley rat.

(ii) *Reporting requirements.* (A) The reproduction and fertility test shall be completed and the final results submitted to EPA within 32 months of the effective date of this final rule.

(B) Progress reports shall be submitted to the Agency every 6 months after the effective date of the final rule.

(2) *Developmental toxicity*—(i) *Required testing.* (A) A test of developmental toxicity shall be conducted with 1,2,4,5-TCB in accordance with § 798.4900 of this chapter.

(B) The route of administration for the developmental toxicity testing for 1,2,4,5-TCB shall be via oral gavage.

(C) Two rodent species shall be used in the study. One shall be the Fischer-344 rat and the second the New Zealand white rabbit.

(ii) *Reporting requirements.* (A) The developmental toxicity testing shall be completed and the final results submitted to the Agency within 16 months of the effective date of the test rule.

(B) Progress reports shall be submitted to the Agency every 6 months after the effective date of the final rule.

(Approved by the Office of Management and Budget under control number 2070-0033)

[51 FR 24667, July 8, 1986, as amended at 52 FR 10378, Apr. 1, 1987; 52 FR 26477, July 15, 1987; 54 FR 27355, June 29, 1989]

### § 799.1250 Cresols.

(a) *Identification of test substances.* (1) *ortho*-Cresol (CAS No. 95-48-7), *meta*-cresol (CAS No. 108-39-4), and *para*-cresol (CAS No. 106-44-5) shall each be tested in accordance with this section.

(2) *ortho-*, *meta-*, and *para*-Cresol of at least 99 percent purity shall be used as the test substance.

(b) *Persons required to submit study plans, conduct tests, and submit data.* (1) All persons who manufacture or process or intend to manufacture or process cresols from the effective date of this rule (June 11, 1986) to the end of the reimbursement period shall submit letters of intent to conduct testing or exemption applications, study plans, and/or shall conduct tests and submit data as specified in this section, Subpart A of this part, and Part 790 of this chapter.

(2) Persons subject to this sectio
are not subject to the requirements
§§ 790.50(a)(2), (5), and (6) and (b
and 790.87(a)(1)(ii) of this chapter.

(3) Persons who notify EPA of the
intent to conduct tests in complianc
with the requirements of this sectio
must submit study plans for thos
tests no later than 30 days before th
initiation of each of those tests.

(4) In addition to the requirement
of § 790.87(a)(2) and (3) of this chap
ter, EPA will conditionally approve ex
emption applications for this rule
EPA has received a letter of intent t
conduct the testing from which ex
emption is sought and EPA has adopt
ed test standards and schedules in
final Phase II test rule.

(c) *Health effects testing*—(1) *Muta
genic effects—chromosomal aberra
tions*—(i) *Required testing.* (A) *I
vitro* cytogenetics tests shall be con
ducted individually with *ortho-*, *meta-*
*and para*-cresol;

(B) An *in vivo* cytogenetics tes
shall be conducted for each isome
which produces a negative result i
the *in vitro* cytogenetics test conduct
ed pursuant to paragraph (c)(1)(i)(A
of this section.

(C) A dominant lethal assay shall b
conducted for each isomer which pro
duces a positive result in either the *i
vitro* or the *in vivo* cytogenetics tes
conducted pursuant to paragraph
(c)(1)(i)(A) and (B) of this section.

(ii) *Test standards.* (A)(*1*) *In vitr
mammalian cytogenetics test.* Thi
test shall be conducted individually
with *ortho-*, *meta-*, and *para*-cresols i
accordance with § 798.5375 of thi
chapter, except for the provisions i
paragraphs (d) (3)(i) and (4) and (6) (i
and (ii).

(*2*) For the purposes of this section
the following provisions also apply:

(*i*) *Type of cells used in the assay
Ortho-*, *meta-*, and *para*-cresols shall
be tested in established cell lines. The
cell lines or strain shall be checked for
*Mycoplasma* contamination.

(*ii*) *Metabolic activation.* The metabolic activation system for this assay
shall be derived from Aroclor-1254 induced rat liver S-9 preparations.

(*iii*) *Test substance—Vehicle. Ortho-*,
*meta-*, and *para*-cresols shall be dis-

solved in DMSO prior to treatment of the cells.

(*iv*) *Exposure concentrations.* At least three concentrations of the test substance over a range adequate to define the response curve shall be tested. The highest test concentration tested with and without metabolic activation shall be 5 milligrams per milliliter or that dose which shows evidence of cytotoxicity or reduced mitotic activity.

(B) (*1*) *In vivo mammalian bone marrow cytogenetics test.* This chromosomal analysis test shall be conducted with each *ortho-*, *meta-*, or *para*-cresol isomer which produces a negative result in the *in vitro* cytogenetics test conducted pursuant to paragraph (c)(1)(i)(A) of this section. This test shall be conducted in accordance with § 798.5385 of this chapter, except for the provisions in paragraphs (d) (3)(i) and (5) (ii) and (iii).

(*2*) For the purposes of this section the following provisions also apply:

(*i*) *Animal selection—Species and strain.* The mouse shall be used. Commonly used laboratory strains should be employed. The test sponsor should provide justification/reasoning for its selection.

(*ii*) *Dose levels.* At least three dose levels shall be used. The highest dose tested shall be the maximum tolerated dose or that producing some indication of cytotoxicity, e.g., partial inhibition of mitosis, or shall be the highest dose attainable.

(*iii*) *Route of administration.* The test substance shall be administered only once by oral gavage.

(C) (*1*) *Rodent dominant-lethal assay.* This assay shall be conducted with *ortho-*, *meta-*, or *para*-cresols in accordance with § 798.5450 of this chapter, except for the provision in paragraphs (d) (3)(i) and (5)(iii) and (e)(1). The rodent dominant-lethal assay shall be conducted for each isomer which produces a positive result in either the in *vitro* or the *in vivo* cytogenetics test conducted pursuant to paragraphs (c)(1)(i) (A) and (B) of this section.

(*2*) For the purposes of this section the following provisions also apply:

(*i*) *Animal selection—Species.* The mouse shall be used. Commonly used laboratory strains should be employed. The test sponsor should provide justification/reasoning for its selection.

(*ii*) *Route of administration.* The test substance shall be administered by oral gavage.

(*iii*) *Test performance.* Each male shall be mated to no more than two, and preferably to only one, female per mating interval. Females shall be left with the males for no longer than 7 days, and mating shall continue for at least 6 weeks.

(iii) *Reporting requirements.* (A) The chromosomal aberration tests shall be completed and the final results submitted to the Agency as follows:

(*1*) The *in vitro* and *in vivo* (conditional) tests shall be completed and the final results submitted to EPA within 12 and 19 months, respectively, of the effective date of the final Phase II test rule.

(*2*) The dominant lethal assay (conditional) within 24 months of the effective date of the final Phase II test rule.

(B) Progress reports shall be submitted to the Agency for the *in vitro* and *in vivo* cytogenetics assays and the dominant lethal assay at 6-month intervals, the first of which is due within 6 months of the effective date of the final Phase II rule.

(2) *Mutagenic effects—gene mutations*—(i) *Required testing.* (A) A DNA damage assay shall be conducted with *meta*-cresol.

(B) A gene mutation in somatic cells assay shall be conducted individually with *meta-* and *para*-cresol.

(C) A sex-linked recessive lethal test in *Drosophila melanogaster* shall be conducted individually with *ortho-* and *para*-cresol.

(D) A sex-linked recessive lethal test in *Drosophila melanogaster* shall be conducted with *meta-* cresol if it produces a positive result in the DNA damage assay or gene mutation in somatic cells assay conducted pursuant to paragraphs (c)(2)(i)(A) and (B) of this section.

(ii) *Test standards.* (A)(*1*) *Unscheduled DNA synthesis in mammalian cells in culture assay.* This assay shall be conducted with *meta*-cresol in accordance with § 798.5550 of this chapter, except for provisions in § 798.5550(d) (3)(i) and (6)(i).

(*2*) For the purposes of this section the following provisions also apply:

(*i*) *Cells—Types of cells used in the assay.* Primary cultures of rat hepatocytes shall be used.

(*ii*) *Test chemical—Vehicle. Meta*-cresol shall be dissolved in DMSO prior to treatment of cells.

(B)(*1*) *Detection of gene mutations in somatic cells in culture.* This assay shall be conducted individually with *meta*- and *para*-cresols in accordance with § 798.5300 of this chapter, except for provisions in § 798.5300(d)(3)(i), (4), (6)(i), and (e)(1).

(*2*) For the purposes of this section the following provisions also apply:

(*i*) *Cells—Type of cells used in the assay.* L5178Y mouse lymphoma cells shall be used. Cells shall be checked for *Mycoplasma* contamination.

(*ii*) *Metabolic activation.* The metabolic activation system shall be derived from the postmitochondrial fraction (S-9) of rat livers pretreated with Aroclor 1254.

(*iii*) *Test chemical—Vehicle. Meta*- and *para*-cresols shall be dissolved in DMSO prior to treatment of the cells. The final concentration of the vehicle shall not interfere with cell viability or growth rate.

(*iv*) *Test performance—Exposure.* Exposure shall be for 4 hours unless a different exposure time is justified by the investigator.

(C) (*1*) *Sex-linked recessive lethal test in Drosophila melanogaster.* This test shall be conducted with *meta*-cresols in accordance with § 798.5275 of this chapter, except for the provisions in § 798.5275(d)(5)(iii). This sex-linked recessive lethal test shall be conducted with *meta*-cresol if it produces a positive result in either one of the assays conducted pursuant to paragraphs (c)(2)(i) (A) and (B) of this section.

(*2*) For the purposes of this section the following provision also applies: *Route of administration.* The oral route of administration shall be used.

(iii) *Reporting requirements.* (A) The genetic toxicity tests shall be completed and final results submitted to the Agency as follows:

(*1*) The unscheduled DNA synthesis in mammalian cells in culture assay within 12 months of the effective date of the final Phase II test rule.

(*2*) The detection of gene mutations in somatic cells in culture assay within 12 months of the effective date of the final Phase II test rule.

(*3*) The sex-linked recessive lethal test in *Drosophila melanogaster*, if required, within 24 months of the effective date of the final Phase II test rule.

(B) Progress reports shall be submitted to the Agency for the unscheduled DNA synthesis in mammalian cells in culture assay, gene mutation in mammalian cells in culture assay, and the *Drosophila* sex-linked recessive lethal test at 6-month intervals, the first of which is due within 6 months of the effective date of the final Phase II rule.

(3) *Mutagenic effects—cellular transformation*—(i) *Required testing.* (A) A Balb/c-3T3 cellular transformation test performed without metabolic activation shall be conducted individually with *meta*- and *para*-cresol.

(B) A Balb/c-3T3 cellular transformation test performed with metabolic activation shall be conducted with each isomer which produces a negative result in the cellular transformation test without metabolic activation conducted pursuant to paragraph (c)(3)(i)(A) of this section.

(C) A Balb/c-3T3 cellular transformation test performed with metabolic activation shall be conducted with *ortho*-cresol.

(ii) *Test standards.* (A) *Morphologic transformation of mammalian cells in culture.* This test shall be conducted individually with *ortho-*, *meta-*, and *para*-cresols in accordance with § 795.285 of this chapter, except for provisions in § 795.285(d)(4).

(B) For the purposes of this section the following provision also applies: *Metabolic activation. Meta*- and *para*-cresol shall initially be tested in this assay performed without metabolic activation. Only if they produce negative results in the assay performed without activation will *meta*- and *para*-cresol then be tested in the assay with metabolic activation. *Ortho*-cresol shall only be tested in this assay performed with metabolic activation.

(iii) *Reporting requirements.* (A) The morphologic transformation of mammalian cells in culture assay shall be completed and final results submitted to EPA within 17 months of the effective date of the final Phase II test rule.

(B) Progress reports shall be submitted to the Agency for the morphologic transformation assay at 6-month intervals, the first of which is due within 6 months of the effective date of the final Phase II rule.

(4) *Developmental toxicity*—(i) *Required testing.* A developmental toxicity study shall be conducted individually with *ortho-*, *meta-*, and *para*-cresol.

(ii) *Test standards.* (A) *Developmental toxicity.* This study shall be conducted individually with *ortho-*, *meta-*, and *para*-cresols in accordance with § 798.4900 of this chapter, except for provisions in § 798.4900(e)(5).

(B) For the purposes of this section the following provision also applies: *Administration of test substance.* The test substance shall be administered by oral gavage.

(iii) *Reporting requirements.* (A) The developmental toxicity study shall be completed and final results submitted to the Agency within 12 months of the effective date of the final Phase II test rule.

(B) Progress reports shall be submitted to the Agency for the developmental toxicity study at 6-month intervals, the first of which is due within 6 months of the effective date of the final Phase II rule.

(5) *Reproductive effects*—(i) *Required testing.* A two-generation reproductive effects study shall be conducted individually with *ortho-*, *meta-*, and *para*-cresol.

(ii) *Test standards.* (A) *Reproduction and fertility effects.* This study shall be conducted individually with *ortho-*, *meta-*, and *para*-cresols in accordance with § 798.4700 of this chapter, except for provisions in § 798.4700(c)(5)(i)(A).

(B) For the purposes of this section the following provision also applies: *Administration of the test substance—Oral studies.* The test substance shall be administered by oral gavage.

(iii) *Reporting requirements.* (A) The reproduction and fertility effects study shall be completed and final results submitted to the Agency within 29 months of the effective date of the final Phase II test rule.

(B) Progress reports shall be submitted to the Agency for the reproduction and fertility effects study at 6-month intervals, the first of which is due within 6 months of the effective date of the final Phase II rule.

(d) *Effective date.* The effective date of the final Phase II rule for cresols is July 6, 1987.

(Information collection requirements have been approved by the Office of Management and Budget under control number 2070-0033)

[51 FR 15782, Apr. 28, 1986, as amended at 52 FR 19087, May 20, 1987; 54 FR 27355, June 29, 1989]

### § 799.1285 Cumene.

(a) *Identification of test substance.* (1) Cumene (isopropylbenzene, CAS No. 98-82-8) shall be tested in accordance with this section.

(2) Cumene of at least 99 percent purity shall be used as the test substance.

(b) *Persons required to submit study plans, conduct tests, and submit data.* All persons who manufacture (including import or byproduct manufacture) or process or intend to manufacture or process cumene, other than as an impurity, after September 9, 1988, to the end of the reimbursement period shall submit letters of intent to conduct testing, submit study plans, conduct tests, and submit data, or submit exemption applications, as specified in this section, Subpart A of this part, and Parts 790 and 792 of this chapter for single-phase rulemaking.

(c) *Health effects*—(1) *Oral and inhalation pharmacokinetic test*—(i) *Required testing.* Pharmacokinetic testing using the oral and inhalation routes shall be conducted with cumene in accordance with § 795.230 of this chapter.

(ii) *Reporting requirements.* (A) The pharmacokinetic testing shall be completed and the final report submitted to EPA within 15 months of the effective date of the final rule.

(B) Interim progress reports shall be submitted to EPA at 6-month intervals beginning 6 months after the effective date of the final rule, until the final report is submitted to EPA.

(2) *Subchronic inhalation toxicity*—(i) *Required testing.* (A) A subchronic inhalation toxicity test shall be conducted with cumene in accordance with § 798.2450 of this chapter except for the provisions of paragraphs (d)(1)(iv), (5), (6), (9), (12)(iii), (13)(i), and (e)(3)(iv)(D) of § 798.2450.

(B) For the purpose of this section, the following provisions also apply.

(*1*) *Animal selection—Numbers.* At least 30 animals (15 males and 15 females) shall be used for each test group.

(*2*) *Exposure conditions.* The animals shall be exposed to the test substance 6 hours per day, 5 days per week for 13 weeks (65 days of exposure).

(*3*) *Observation of animals.* Animals shall be weighed weekly, and their food and water consumption shall also be measured weekly.

(*4*) *Gross pathology.* The following additional organs shall be preserved in a suitable medium for future histopathological examination: The vas deferens, the oviducts, and the vagina.

(*5*) *Histopathology.* The accessory genital organs (epididymis), prostate, seminal vesicles) and the vagina shall be examined histopathologically. In addition, preparations of testicular and associated reproductive organ samples for histology shall follow the recommendations of Lamb and Chapin (1985) under paragraph (f)(1) of this section, or an equivalent procedure, with particular attention directed toward achieving optimal quality in the fixation and embedding, and including an evaluation of the spermatogenic pattern. Spermatid counts shall be performed as described by Johnson et al. (1980) and Blazak et al. (1985) under paragraphs (d) (2) and (3) of this section or an equivalent procedure. Epididymal sperm count and sperm morphology shall also be done.

(*6*) *Test report—Individual animal data.* The specific test report information shall include "Food and water consumption data."

(ii) *Reporting requirements.* (A) The subchronic toxicity test shall be completed and the final report submitted to EPA within 15 months of the effective date of the final rule.

(B) Interim progress reports shall be submitted to EPA at 6-month intervals beginning 6 months after the effective date of the final rule, until the final report is submitted to EPA.

(3) *Inhalation developmental toxicity*—(i) *Required testing.* An inhalation developmental toxicity test shall be conducted with cumene in accordance with § 798.4350 of this chapter.

(ii) *Reporting requirements.* (A) The inhalation developmental toxicity test shall be completed and the final report submitted to EPA within 15 months of the effective date of the final rule.

(B) Interim progress reports shall be submitted to EPA at 6-month intervals beginning 6 months after the effective date of the final rule, until the final report is submitted to EPA.

(3) *Neurotoxicity*—(i) *Required testing.* (A) Neurotoxicity tests shall be conducted with cumene by inhalation in accordance with §§ 798.6050, 798.6200, and 798.6400 of this chapter. Each test shall be performed for a period of 90 days.

(ii) *Reporting requirements.* (A) The neurotoxicity tests shall be completed and the final reports submitted to EPA within 15 months of the effective date of the final rule.

(B) Interim progress reports for each test shall be submitted to EPA at 6-month intervals beginning 6 months after the effective date of the final rule, until the applicable final report is submitted to EPA.

(5) *Two-generation reproductive effects*—(i) *Required testing.* A two-generation reproductive effects test shall be conducted with cumene in accordance with § 798.4700 of this chapter if either the gross or histopathological evaluation of the reproductive tissues in male or female exposed animals from the subchronic exposure test specified in paragraph (c)(2) of this section shows adverse effects or if significant alteration in reproductive organ weights occurs in the subchronic exposure test which can be related to exposure to cumene. EPA will hold a public program review, following submission of the subchronic toxicity test, to decide whether the two-

generation reproductive effects test is to be required. If required, the test should be conducted using the oral route of exposure.

(ii) *Reporting requirements.* (A) The two-generation reproductive effects test shall be completed and the final report submitted to EPA within 29 months following EPA's notification to the test sponsor, through certified letter or FEDERAL REGISTER notice, that testing shall be initiated.

(B) Interim progress reports shall be submitted to EPA at 6-month intervals beginning 6 months after the date of EPA's notification to the test sponsor that testing shall be initiated, until the final report is submitted to EPA.

(d) *Environmental effects*—(1) *Aquatic acute toxicity*—(i) *Required testing.* Saltwater and freshwater invertebrate and vertebrate tests, in a flow-through system, shall be conducted with cumene on the following organisms: *Daphnia magna*, to be conducted in accordance with § 797.1300 of this chapter; *Mysidopsis bahia* to be conducted in accordance with § 797.1930 of this chapter; and *Salmo gairdneri* and *Cyprinodon variegatus* to be conducted in accordance with § 797.1400 of this chapter. The total and dissolved (e.g. filtered) concentrations of the test substance shall be measured in each test chamber and the delivery chamber before the test and in each test chamber at 0, 24, and 48 hours (*Daphnia magna*) and 0, 48, and 96 hours (*Mysidopsis bahia, Salmo gairdneri*, and *Cyprinodon variegatus*) to ascertain whether it is in solution.

(ii) *Reporting requirements.* (A) The acute toxicity tests shall be completed and the final reports submitted to EPA within 12 months of the effective date of the final rule.

(B) An interim progress report for each acute test shall be submitted to EPA 6 months after the effective date of the final rule.

(2) *Aquatic chronic toxicity*—(i) *Required testing.* Aquatic chronic toxicity tests, in a flow-through system, shall be conducted with cumene on *Daphnia magna*, in accordance with § 797.1330 of this chapter, and *Mysidopsis bahia*, in accordance with § 797.1950 of this chapter, if the results of the acute toxicity tests conducted for those species under paragraph (d)(1) of this section show $EC_{50}$ or $LC_{50}$ of less than or equal to 1 mg/L. The total and dissolved (e.g. filtered) concentrations of the test substance shall be measured in each test chamber and the delivery chamber before the test and in each test chamber and the delivery chamber at 0, 7, 14, and 21 days to ascertain whether it is in solution.

(ii) *Reporting requirements.* (A) The chronic toxicity tests, if required under paragraph (d)(2)(i) of this section, shall be completed and the final reports submitted to EPA within 24 months of the effective date of the final rule.

(B) An interim progress report for each chronic test shall be submitted to EPA 18 months after the effective date of the final rule.

(3) *Aquatic early life stage toxicity*—(i) *Required testing.* Aquatic early life stage toxicity tests, in a flow-through system, shall be conducted with cumene on *Salmo gairdneri* and *Cyprinodon variegatus*, in accordance with § 797.1600 of this chapter, if the results of the acute toxicity tests conducted for those species under paragraph (d)(1) of this section show $LC_{50}$ of less than or equal to 1 mg/L.

(ii) *Reporting requirements.* (A) The early life stage toxicity tests, if required under paragraph (d)(3) of this section, shall be completed and the final reports submitted to EPA within 24 months of the effective date of the final rule.

(B) An interim progress report for each test shall be submitted to EPA 18 months after the effective date of the final rule.

(e) *Chemical fate*—(1) *Biodegradation*—(i) *Required testing.* Biodegradation testing in an aquatic system shall be conducted with cumene in accordance with the method described in an article by Bourquin et al. entitled "An Artificial Microbial Ecosystem for Determining Effects and Fate of Toxicants in a Salt-Marsh Environment," reprinted from Vol. 18 of the Society of Industrial Microbiology's *Developments in Industrial Microbiology*, Chapter 11, 1977, which is incorporated by reference. The method is available for public inspection at the Office of the Federal Register, Rm. 8301, 11th and L St., NW., Washington, DC 20408, and copies may be obtained from the EPA TSCA Public Docket Office (TS-793), Rm. G-004 Northeast Mall, 401 M St., SW., Washington, DC 20460. This incorporation by reference was approved by the Director of the Federal Register in accordance with 5 U.S.C. 522(a) and 1 CFR Part 51. The method is incorporated as it exists on the effective date of this rule and a notice of any change to the method will be published in the FEDERAL REGISTER.

(ii) *Reporting requirements.* (A) The biodegradation test in an aquatic system shall be completed and the final report submitted to EPA within 12 months of the effective date of the final rule.

(B) An interim progress report shall be submitted to EPA 6 months after the effective date of the final rule.

(2) *Volatilization*—(i) *Required testing.* A test for volatilization from aquatic system shall be conducted with cumene in accordance with the method described in an article by Smith et al. entitled "Prediction of the Volatilization Rates of High-Volatility Chemicals from Natural Water Bodies," published in Vol. 14, Number 11, of the American Chemical Society's *Environmental Science & Technology,* 1980, which is incorporated by reference. The method is available for public inspection at the Office of the Federal Register, Rm. 8301, 11th and L St., NW., Washington, DC 20408, and copies may be obtained from the EPA TSCA Public Docket Office (TS-793), Rm. G-004 Northeast Mall, 401 M St., SW., Washington, DC 20460. This incorporation by reference was approved by the Director of the Federal Register in accordance with 5 U.S.C. 522(a) and 1 CFR Part 51. The method is incorported as it exists on the effective date of this rule and a notice of any change to the method will be published in the FEDERAL REGISTER.

(ii) *Reporting requirements.* (A) The volatilization test in an aquatic system shall be completed and the final report submitted to EPA within 12 months of the effective date of the final rule.

(B) An interim progress report shall be submitted to EPA 6 months after the effective date of the final rule.

(f) *References.* For additional background information, the following references should be consulted:

(1) Lamb, J.C. and Chapin, R.E. "Experimental models of male reproductive toxicology," *Endocrine Toxicology.* Eds. J.A. Thomas, K.S. Korach, J.A. McLachlan. New York, NY: Raven Press, pp. 85-115 (1985).

(2) Johnson, L., Petty, C.S., and Neaves, W.B. "A comparative study of daily sperm production and testicular composition in humans and rats," *Biology of Reproduction,* 22:1233-1241 (1980).

(3) Blazak, W.F., Ernest, T.L., and Stewart, B.E. "Potential indicators of reproductive toxicity: Testicular sperm production and epididymal sperm number, transit time and motility in Fischer 344 rats," *Fundamental and Applied Toxicology,* 5:1097-1103 (1985).

(g) *Effective date.* (1) The effective date of this final rule for cumene is September 9, 1988.

(2) The guidelines and other test methods cited in this section are referenced here as they exist on September 9, 1988.

(Information collection requirements have been approved by the Office of Management and Budget under control number 2070-0033)

[53 FR 28204, July 27, 1988]

### § 799.1550 1,2-Dichloropropane.

(a) *Identification of test substance.* (1) 1,2-Dichloropropane (CAS No. 78-87-5) shall be tested in accordance with this section.

(2) 1,2-Dichloropropane of at least 99 percent purity shall be used as the test substance.

(b) *Persons required to submit study plans, conduct tests, and submit data.* (1) All persons who manufacture or process 1,2-dichloropropane, other than as an impurity, from October 23, 1986 to the end of the reimbursement period, shall submit letters of intent to conduct testing or exemption applications, conduct tests, and submit data as specified in paragraphs (c)(1), (c)(2), (c)(3), and (c)(4), and (d) of this section, Subpart A of this part, and Parts 790 and 792 of this chapter for two-phase rulemaking.

(2) Persons subject to this section are not subject to the requirements of §§ 790.50(a)(2), (5), (6), and (b)(2) and (4), and 790.87(a)(1)(ii) of this chapter.

(3) Persons who notify EPA of their intent to conduct tests in compliance with the requirements of this section must submit plans for those tests no later than 45 days before the initiation of each of those tests.

(4) In addition to the requirements of § 790.87(a)(2) and (3) of this chapter, EPA will conditionally approve exemption applications for this rule if EPA has received a letter of intent to conduct the testing from which exemption is sought and EPA has adopted test standards and schedules in a final rule.

(5) All persons who manufacture or process 1,2-dichloropropane, other than as an impurity, from November 18, 1987 to the end of the reimbursement period, shall submit letters of intent to conduct testing or submit exemption applications, conduct tests, and submit data as specified in paragraph (c)(5) of this section, Subpart A of this part, and Parts 790 and 792 of this chapter for single-phase rulemaking.

(c) *Health effects testing*—(1) *Neurotoxicity*—(i) *Required testing.* The following neurotoxicity testing shall be conducted for 1,2-dichloropropane:

(A) A neuropathology test.

(B) A motor activity test.

(C) A functional observational battery.

(ii) *Test standards.* The neurotoxicity testing with 1,2-dichloropropane, consisting of a neuropathology test, a motor activity test, and a functional observational battery, shall be conducted in accordance with §§ 798.6400, 798.6200, and 798.6050 of this chapter, respectively, using the oral route of exposure. The animals shall be dosed with DCP for a minimum of 5 days per week, over a period of at least 90 days.

(iii) *Reporting requirements* (A) The neurotoxicity tests shall be completed and the final reports submitted to EPA within 1 year of the effective date of the final Phase II rule.

(B) An interim progress report shall be submitted to EPA 6 months after the effective date of the final Phase II rule.

(2) *Mutagenic effects*—(i) *Required testing.* A dominant lethal assay shall be conducted with 1,2-dichloropropane.

(ii) *Test standards.* The dominant lethal assay with 1,2-dichloropropane shall be conducted in accordance with § 798.5450 of this chapter.

(iii) *Reporting requirements.* (A) The dominant lethal assay shall be completed and the final report submitted to EPA within 18 months of the effective date of the final Phase II rule.

(B) An interim progress report shall be submitted to EPA 6 months after the effective date of the final Phase II rule.

(3) *Developmental toxicity*—(i) *Required testing.* A developmental toxicity test shall be conducted with 1,2-dichloropropane.

(ii) *Test standard.* The developmental toxicity test with 1,2-dichloropropane shall be conducted in accordance with § 798.4900 of this chapter, using the oral route of exposure.

(iii) *Reporting requirements.* (A) The developmental toxicity test shall be completed and the final report submitted to EPA within 18 months of the effective date of the final Phase II rule.

(B) Interim progress reports shall be submitted to EPA at 6-month intervals, beginning 6 months after the effective date of the final Phase II rule and ending with the submission of the Final Test Report.

(4) *Reproductive effects*—(i) *Required testing.* A two-generation reproductive effects study shall be conducted with 1,2-dichloropropane.

(ii) *Test standard.* The two-generation reproductive effects testing with 1,2-dichloropropane shall be conducted in accordance with § 798.4700 of this chapter, using the oral route of exposure.

(iii) *Reporting requirement.* (A) The two-generation reproductive effects test shall be completed and the final report submitted to EPA within 29 months of the effective date of the final Phase II rule.

(B) Interim progress reports shall be submitted to EPA at 6-month intervals, beginning 6 months after the effective date of the final Phase II rule and ending with the submission of the Final Test Report.

(5) *Pharmacokinetic studies*—(i) *Required testing.* An oral and inhalation pharmacokinetic test shall be conducted with 1,2-dichloropropane.

(ii) *Test standard.* (A) The oral and inhalation pharmacokinetic testing with 1,2-dichloropropane shall be conducted in accordance with § 795.230 of this chapter, except for the provisions in paragraphs (c)(2) (i), (ii) (A) and (B), (iii) (C) and (D), and (3)(ii) of § 795.230.

(B) For the purpose of this section, the following provisions also apply:

(*1*) *Test Substance.* The studies require the use of both non-radioactive and $^{14}$C-labeled test substance. The non-radioactive test substance shall be at least 99 percent pure, while the radiochemical purity of the $^{14}$C-labeled test substance may be slightly less than 99 percent. Both preparations are needed to investigate the provisions of paragraph (a)(2) of this section. The use of $^{14}$C-test substance is recommended for the provisions in paragraph (a)(1), (2), and (3) of this section in order to facilitate the work, improve the reliability of quantitative determinations, and increase the probability of observing previously unidentified metabolites.

(*2*) *Oral study.* At least two doses shall be used in the study, a "low" and "high" dose. When administered orally, the "high" doses should induce some overt toxicity such as weight loss. If data from prior repeated dosing studies is utilized to select the "high" dose, overt toxicity need not be elicited in this exposure group. The "low" dose shall not induce observable effects attributable to the test substance. Oral dosing shall be performed by gavage using an appropriate vehicle.

(*3*) *Inhalation study.* Three concentrations shall be used in the study. Upon exposure, two higher concentrations should ideally induce some overt symptoms of toxicity, although the intermediate concentration may be excluded from this condition. If data from prior repeated dosing studies is utilized to select the high dose, overt toxicity need not be elicited in this exposure group. The lowest concentration shall not induce observable effects attributable to the test substance.

(*4*) *Collection of excreta.* After oral administration (Groups A and B) and inhalation exposure (Groups F through H) the rats shall be placed in individual metabolic cages and excreta (urine, feces and expired air) shall be collected from 0 to 24 hours and from 24 to 48 hours after dosing and, if necessary, daily thereafter until at least 90 percent of the dose has been excreted or until 7 days after dosing, whichever occurs first.

(*5*) *Kinetic studies.* Groups C through E should be used to determine the concentration of the test substance in blood at 0, 5, 10, 15, and 30 minutes, and at 1, 2, 4, 8, 16, 24, and 48 hours after initiation of inhalation exposure. If experimentally feasible, blood obtained from the $^{14}$C-exposed rats from Groups F through H may be used to determine the test substance concentrations.

(*6*) *Biotransformation after oral and inhalation exposure.* Appropriate qualitative and quantitative methods shall be used to assay urine specimens collected from each rat in Groups A and B and F through H. The radiometric analyses of urine, feces and expired air should be conducted individually for each rat, but samples from each rat per time point may be pooled for analytical determination of parent compound and metabolite identification. Metabolite identification shall be attempted for those routes of excretion which contain greater than 10 percent of the oral dose or, in the inhalation study, greater, than 10 percent of the body burden at the end of exposure.

(iii) *Reporting requirements.* (A) The pharmacokinetic test shall be completed and the final report submitted to EPA within 17 months of the effective date of the final single-phase pharmacokinetics rule.

(B) An interim progress report shall be submitted to EPA 6 months after the effective date of the final single-phase rule.

(d) *Environmental effects testing*—(1) *Mysid acute toxicity*—(i) *Required testing.* A mysid shrimp acute toxicity test shall be conducted with 1,2-dichloropropane.

(ii) *Test standard.* The mysid shrimp acute toxicity test with 1,2-dichloropropane shall be conducted as a flow-through test with measured concentrations using *Mysidopsis bahia* in accordance with § 797.1930 of this chapter.

(iii) *Reporting requirements.* (A) The mysid acute toxicity test shall be completed and the final report submitted to EPA within 1 year of the effective date of the final Phase II rule.

(B) An interim progress report shall be submitted to EPA 6 months after the effective date of the final Phase II rule.

(2) *Algal acute toxicity*—(i) *Required testing.* An algal acute toxicity test shall be conducted with 1,2-dichloropropane.

(ii) *Test standard.* (A) The algal acute toxicity tests with 1, 2-dichloropropane shall be conducted with marine and freshwater algae using systems that control for 1,2-dichloropropane evaporation in accordance with § 797.1050 of this chapter, except for the provisions in paragraph (c)(1)(ii), (3)(iii), (4)(iv), (6)(i)(B), (d)(3)(ii) and (iii), (e)(4) and (5) of § 797.1050.

(B) For the purpose of this section, the following provisions shall also apply to the algal acute toxicity tests:

(*1*) At 48, 72, 96, and 120 hours, enumerate the algal cells in all containers to determine the inhibition or stimulation of growth in test containers compared to controls. Use data to define the concentration-response curve, and calculate the EC$_{10}$, EC$_{50}$, and EC$_{90}$ values.

(*2*) The test is performed once for each of the recommended algal species or selected alternates. Test chambers should contain equal volumes of test solution and approximately $1 \times 10^4$ *Selenastrum* cells/ml or $7.7 \times 10^4$ *Skeletonema* cells/ml of test solution. The algae should be exposed to each concentration of test chemical for up to 120 hours. The exposure period may be shortened if data suitable for the purposes of the range-finding tests can be obtained in less time.

(*3*) The test begins when algae from 7 to 10-day-old stock cultures are placed in the test chambers containing test solutions having the appropriate concentrations of the test substance. At the end of 120 hours, the algal growth response (number or weight of algal cells/ml) in all test containers and controls should be determined by an indirect (spectrophotometry, electronic cell counters, dry weight, etc.) or a direct (actual microscopic cell count) method. Indirect methods should be calibrated by a direct microscopic count. The percentage inhibition of stimulation of growth for each concentration, EC$_{10}$, EC$_{50}$, EC$_{90}$, and the concentration-response curves are determined from these counts.

(*4*) At the end of the test and after aliquots have been removed for algal growth-response determinations, microscopic examination, mortal staining, or subculturing, the replicate test containers for each chemical concentration may be pooled into one sample. An aliquot of the pooled sample may then be taken and the concentration of test chemical determined. In addition, the concentration of test chemical associated with the algae alone should be determined. Separate and concentrate the algal cells from the test solution by centrifuging or filtering the remaining pooled sample and measure the test substance concentration in the algal-cell concentrate. The concentrations associated with the algae do not have to be measured if data are provided that demonstrate that substantive amounts of the test substance are lost during transfer of algae to centrifuge tubes or during centrifugation.

(*5*) Test chambers containing *Selenastrum* shall be illuminated continuously and those containing *Skeletonema* shall be provided a 14-hour light and 10-hour dark photoperiod under fluorescent lamps providing 300±25uEin/m$^2$ sec (approximately 400 ft-c) measured adjacent to the test chambers at the level of test solution.

(*6*) Stock algal cultures should be shaken twice daily by hand. Test containers may be shaken by hand or placed on a rotary shaking apparatus and oscillated at approximately 100 cycles/minute for *Selenastrum* and at approximately 60 cycles/minute for *Skeletonema* during the test. The rate of oscillation should be determined at least once daily during testing.

(*7*) The number of algal cells per milliliter in each treatment and control and the method used to derive these values at the beginning, 48, 72, and 96 hours, and end of the test; the percentage of inhibition or stimulation of growth relative to controls; and other adverse effects in the control and in each treatment.

(*8*) The 120-hour $EC_{10}$, $EC_{50}$, and $EC_{90}$, values, and when sufficient data have been generated, the 48, 72, and 96 hour $LC_{50}$'s and 95 percent confidence limits, the methods used to derive these values, the data used to define the shape of the concentration-response curve and the goodness-of-fit determination.

(iii) *Reporting requirements.* (A) The algal acute toxicity tests shall be completed and the final report submitted to EPA within 1 year of the effective date of the final Phase II rule.

(B) An interim progress report shall be submitted to EPA 6 months after the effective date of the final Phase II rule.

(3) *Daphnid chronic toxicity*—(i) *Required testing.* A daphnid chronic toxicity test shall be conducted with 1,2-dichloropropane.

(ii) *Test standard.* The daphnid chronic toxicity test with 1,2-dichloropropane shall be conducted as a flowthrough test using *Daphnia magna* in accordance with § 797.1330 of this chapter.

(iii) *Reporting requirements.* (A) The daphnid chronic toxicity test shall be completed and the final report submitted to EPA within 1 year of the effective date of the final Phase II rule.

(B) An interim progress report shall be submitted to EPA 6 months after the effective date of the final Phase II rule.

(4) *Mysid shrimp chronic toxicity*—(i) *Required testing.* A mysid shrimp chronic toxicity test shall be conducted with 1,2-dichloropropane.

(ii) *Test standard.* The mysid shrimp chronic toxicity test with 1,2-dichloropropane shall be conducted as a flowthrough test using *Mysidopsis bahia* in accordance with § 797.1950 of this chapter.

(iii) *Reporting requirements.* (A) The mysid chronic toxicity test shall be completed and the final report submitted to EPA within 1 year of the effective date of the final Phase II rule.

(B) An interim progress report shall be submitted to EPA 6 months after the effective date of the final Phase II rule.

(e) *Effective date.* The effective date of the final Phase II rule and the final single-phase pharmacokinetics rule for 1,2-dichloropropane is November 18, 1987.

(Information collection requirements approved by the Office of Management and Budget under control number 2070-0033)

[51 FR 32087, Sept. 9, 1986, as amended at 52 FR 37144, Oct. 5, 1987; 54 FR 27355, June 29, 1989]

### § 799.1560 Diethylene glycol butyl ether and diethylene glycol butyl ether acetate.

(a) *Identification of test substances.* (1) Diethylene glycol butyl ether (DGBE), CAS Number 112-34-5, and diethylene glycol butyl ether acetate (DGBA), CAS Number 124-17-4, shall be tested in accordance with this section.

(2) DGBE of at least 95 percent purity and DGBA of at least 95 percent purity shall be used as the test substances.

(b) *Persons required to submit study plans, conduct tests, and submit data.* All persons who manufacture (including import) or process or intend to manufacture or process DGBE and/or DGBA, other than as an impurity, after April 11, 1988, to the end of the reimbursement period shall submit letters of intent to conduct testing, submit study plans and conduct tests, and submit data, or submit exemption applications as specified in this section, Subpart A of this part, and Parts 790 and 792 of this chapter for single-phase rulemaking. Persons who manufacture or process DGBE are subject to the requirements to test DGBE in this section. Only persons who manufacture or process DGBA are subject to the requirements to test DGBA in this section.

(c) *Health effects testing*—(1) *Subchronic toxicity*—(i) *Required testing.*

(A) A 90-day subchronic toxicity test of DGBE shall be conducted in rats by dermal application in accordance with § 798.2250 of this chapter except for the provisions in paragraphs (e)(9)(iv), (10)(i)(A) and (ii)(B), (11) (ii) and (iii), and (12)(i) of § 798.2250.

(B) For the purpose of this section, the following provisions also apply:

(*1*) A satellite group to evaluate fertility shall be established. Control males shall be cohabited with control females, and males and females administered the high dose shall be cohabited. Endpoints to be evaluated shall include percent mated; percent pregnant; length of gestation; litter size; viability at birth, on Day 4, and weaning, on Day 21; sex of the offspring; and litter weights at birth and Days 4, 7, 14, and 21. Litters shall be standardized on day 4 in accordance with the reproductive and fertility effects guideline, § 798.4700(c)(6)(iv) of this chapter. Gross examinations shall be made at least once each day and physical or behavioral anomalies in the dam or offspring shall be recorded. At weaning, dams shall be sacrificed and examined for resorption sites indicative of post-implantation loss. An additional 20 males and 40 females will have to be added to the subchronic study for this test. If the animals in the high dose group exhibit marked toxicity (e.g. greater than 20 percent weight loss), then the fertility tests shall be conducted in the next highest dose group.

(*2*) Cage-side observations shall include, but not be limited to, changes in skin and fur; eyes and mucous membranes; respiratory, circulatory autonomic, and central nervous systems; somatomotor activity; and behavior pattern. In addition a daily examination for hematuria shall be done.

(*3*) Certain hematology determinations shall be carried out at least three times during the test period: Just prior to initiation of dosing (baseline data), after approximately 30 days on test, and just prior to terminal sacrifice at the end of the test period. Hematology determinations which are appropriate to all studies: Hematocrit, hemoglobin concentration, erythrocyte count, total and differential leucocyte count, mean corpuscular volume, and a platelet count.

(*4*) Urinalyses shall be done at least three times during the test period: Just prior to initiation of dosing (baseline data), after approximately 30 days into the test, and just prior to terminal sacrifice at the end of the test period. The animals shall be kept in metabolism cages, and the urine shall be examined microscopically for the presence of erythrocytes and renal tubular cells, in addition to measurement of urine volume, specific gravity, glucose, protein/albumin, and blood.

(*5*) The liver, kidney, adrenals, brain, gonads, prostate gland, epididymides, seminal vesicles, and pituitary gland shall be weighed wet, as soon as possible after dissection, to avoid drying.

(*6*) The following organs and tissues, or representative samples thereof, shall be preserved in a suitable medium for possible future histopathological examination: All gross lesions; lungs—which should be removed intact, weighed, and treated with a suitable fixative to ensure that lung structure is maintained (perfusion with the fixative is considered to be an effective procedure); nasopharyngeal tissues; brain—including sections of medulla/pons, cerebellar cortex, and cerebral cortex; pituitary; thyroid/parathyroid; thymus; trachea; heart; sternum with bone marrow; salivary glands; liver; spleen; kidneys; adrenals; pancreas; gonads; uterus; oviducts; vagina; vas deferens; accessory genital organs (epididymis, prostate, and, if present, seminal vesicles); aorta; (skin); gall bladder (if present); esophagus; stomach; duodenum; jejunum; ileum; cecum; colon; rectum; urinary bladder; representative lymph node; (mammary gland); (thigh musculature); peripheral nerve; (eyes); (femur—including articular surface); (spinal cord at three levels—cervical, midthoracic, and lumbar); and (zymbal and exorbital lachrymal glands).

(*7*) (*i*) Full histopathology on normal and treated skin and on organs and tissues listed in paragraph (c)(1)(i)(B)(*6*) of this section, as well as the accessory genital organs (epididymides, prostate, seminal vesicles) and the vagina, of all animals in the control and high dose groups.

(*ii*) The integrity of the various cell stages of spermatogenesis shall be determined, with particular attention directed toward achieving optimal quality in the fixation and embedding; preparations of testicular and associated reproductive organ samples for histology should follow the recommendations of Lamb and Chapin (1985) under paragraph (d)(1) of this section, or an equivalent procedure. Histological analyses shall include evaluations of the spermatogenic cycle, i.e., the presence and integrity of the 14 cell stages. These evaluations should follow the guidance provided by Clermont and Perey (1957) under paragraph (d)(2) of this section. Information shall also be provided regarding the nature and level of lesions observed in control animals for comparative purposes.

(*iii*) Data on female cyclicity shall be obtained by performing vaginal cytology over the last 2 weeks of dosing; the cell staging technique of Sadleir (1978) and the vaginal smear method in Hafez (1970) under paragraphs (d)(3) and (7) of this section or equivalent methods should be used. Data should be provided on whether the animal is cycling and the cycle length.

(*iv*) The ovary shall be serially sectioned with a sufficient number of sections examined to adequately detail oocyte and follicular morphology. The methods of Mattison and Thorgiersson (1979) and Pederson and Peters (1968) under paragraphs (d) (4) and (5) of this section may provide guidance. The strategy for sectioning and evaluation is left to the discretion of the investigator, but shall be described in detail in the study plan and final report. The nature and background level of lesions in control tissue shall also be noted.

(ii) *Reporting requirements.* (A) The subchronic test shall be completed and the final report submitted to EPA within 15 months of the effective date of the final test rule.

(B) Progress reports shall be submitted to EPA every 6 months, beginning 6 months from the effective date of the final rule until submission of the final report to EPA.

(2) *Neurotoxicity/behavioral effects*—(i) *Required testing*—(A) (*1*) *Functional observational battery.* A functional observational battery shall be performed in the rat by dermal application of DGBE for a period of 90 days according to § 798.6050 of this chapter except for the provisions in paragraphs (b)(1), (d)(4)(ii), (5), and (8)(ii)(E) of § 798.6050.

(*2*) For the purpose of this section, the following provisions also apply:

(*i*) *Definition.* Neurotoxicity is any adverse acute and/or lasting effect on the structure or function of the central and/or peripheral nervous system related to exposure to a chemical substance.

(*ii*) *Lower doses.* The data from the lower doses shall show either graded dose-dependent effects in at least two of all the doses tested including the highest dose, or no neurotoxic (behavioral) effects at any dose tested.

(*iii*) *Duration and frequency of exposure.* Animals shall be exposed for 6 hours/day, 5 days/week for a 90-day period.

(*iv*) *Sensory function.* A simple assessment of sensory function (vision, audition, pain perception) shall be made. Marshall et al. (1971) in § 798.6050(*f*)(8) of this chapter have described a neurologic exam for this purpose; these procedures are also discussed by Deuel (1977), under § 798.6050(f)(4) of this chapter. Irwin (1968) under § 798.6050(f)(7) of this chapter described a number of reflex tests intended to detect gross sensory deficits. Many procedures have been developed for assessing pain perception (e.g., Ankier (1974) under § 798.6050(f)(1); D'Amour and Smith (1941) under § 798.6050(f)(3); and Evans (1971) under § 798.6050(f)(6) of this chapter.

(B)(*1*) *Motor activity.* A motor activity test shall be conducted in the rat by dermal application of DGBE for a period of 90 days according to § 798.6200 of this chapter except for the provisions in paragraphs (c), (d)(3)(ii), (4)(ii), (5), (8)(i), and (iii) of § 798.6200.

(*2*) For the purpose of this section, the following provisions also apply:

(*i*) *Principle of the test method.* The test substance is administered to several groups of experimental animals, one dose being used per group. Measurements of motor activity are made. Where possible, the exposure levels at which significant changes in motor activity are produced are compared to those levels which produce toxic effects not originating in the central and/or peripheral nervous system.

(*ii*) *Positive control data.* Positive control data are required to document the sensitivity of the activity measuring device and testing procedure. These data should demonstrate the ability to detect increases or decreases in activity and to generate a dose-effect curve or its equivalent using three values of the dose or equivalent independent variable. A single administration of the dose (or equivalent) is sufficient. It is recommended that chemical exposure be used to collect positive control data. Positive control data shall be collected at the time of the test study unless the laboratory can demonstrate the adequacy of historical data for this purpose.

(*iii*) *Lower doses.* The data from the lower doses shall show either graded dose-dependent effects in at least two of all the doses tested including the highest dose, or no neurotoxic (behavioral) effects at any dose tested.

(*iv*) *Duration and frequency of exposure.* Animals shall be exposed for 6 hours/day, 5 days/week for a 90-day period.

(*v*) *General.* Motor activity shall be monitored by an automated activity recording apparatus. The device used shall be capable of detecting both increases and decreases in activity, i.e. baseline activity as measured by the device shall not be so low as to preclude decreases nor so high as to preclude increases. Each device shall be tested by a standard procedure to ensure, to the extent possible, reliability of operation across devices and across days for any one device. In addition, treatment groups shall be balanced across devices. Each animal shall be tested individually. The test session shall be long enough for motor activity to approach asymptotic levels by the last 20 percent of the session for most treatments and for the session control animals. All sessions should be of the same duration. Treatment groups shall be counter-balanced across test times. Effort should be made to ensure that variations in the test conditions are minimal and are not systematically related to treatment. Among the variables which can affect motor activity are sound level, size and shape of the test cage, temperature, relative humidity, lighting conditions, odors, use of home cage or novel test cage, and environmental distractions. Tests shall be executed by an appropriately trained individual.

(*vi*) *Subchronic.* All animals shall be tested prior to initiation of exposure and at 30±4, 60±4, and 90±4 days during the exposure period. Testing shall occur prior to the daily exposure. Animals shall be weighed on each test day and at least once weekly during the exposure period.

(C)(*1*) *Neuropathology.* A neuropathology test shall be conducted in the rat by dermal application of DGBE for a period of 90 days according to § 798.6400 of this chapter except for the provisions in paragraphs (d)(4)(ii), (5), (8)(iv)(C), and (E)(*2*) of § 798.6400.

(*2*) For the purpose of this section, the following provisions also apply:

(*i*) *Lower doses.* The data from the lower doses shall show either graded dose-dependent effects in at least two of all the doses tested including the highest dose, or no neurotoxic (behavioral) effects at any dose tested.

(*ii*) *Duration and frequency of exposure.* Animals shall be exposed for 6 hours/day, 5 days/week for a 90-day period.

(*iii*) *Clearing and embedding.* After dehydration, tissue specimens shall be cleared with xylene and embedded in paraffin or paraplast except for the sural nerve which should be embedded in plastic. Multiple tissue specimens (e.g. brain, cord, ganglia) may be embedded together in one single block for sectioning. All tissue blocks shall be labeled to provide unequivocal identification. A method for plastic embedding is described by Spencer et al. in paragraph (d)(6) of this section.

(*iv*) *Special stains.* Based on the results of the general staining, selected sites and cellular components shall be further evaluated by the use of specific techniques. If hematoxylin and eosin screening does not provide such information, a battery of stains shall be used to assess the following components in all appropriate required samples: Neuronal body (e.g., Einarson's gallocyanin), axon (e.g., Bodian), myelin sheath (e.g., Kluver's Luxol Fast Blue), and neurofibrils (e.g., Bielchosky). In addition, peripheral nerve fiber teasing may be used. Detailed staining methodology is available in standard histotechnological manuals such as Armed Forces Institute of Pathology (AFIP) (1968) under § 798.6400(f)(1), Ralis et al. (1973) under § 798.6400(f)(5), and Chang (1979) under § 798.6400(f)(2) of this chapter. The nerve fiber teasing technique is discussed in Spencer and Schaumberg (1980) under § 798.6400(f)(6) of this chapter. A section of normal tissue shall be included in each staining to assure that adequate staining has occurred. Any changes shall be noted and representative photographs shall be taken. If a lesion(s) is observed, the special techniques shall be repeated in the next lower treatment group until no further lesion is detectable.

(ii) *Reporting requirements.* (A) The neurotoxicity/behavioral tests required under paragraph (c)(2) of this section shall be completed and the final reports submitted to EPA within 15 months of the effective date of the final rule.

(B) Interim progress reports shall be submitted to EPA at 6-month intervals, beginning 6 months from the effective date of the final rule until submission of the applicable final report to EPA.

(3) *Developmental neurotoxicity*—(i) *Required testing.* A developmental neurotoxicity test of DGBE shall be conducted after a public program review of the Tier I data from the functional observational battery, motor activity, and neuropathology tests in paragraph (c)(2) of this section, and the reproductive tests in paragraph (c)(1) of this section, and if EPA issues a FEDERAL REGISTER notice or sends a certified letter to the test sponsor specifying that the testing shall be initiated. The test shall be performed in rats in accordance with § 795.250 of this chapter.

(ii) *Reporting requirements.* (A) The developmental neurotoxicity test shall be completed and the final report submitted to EPA within 15 months of EPA's notification of the test sponsor by certified letter or FEDERAL REGISTER notice under paragraph (c)(3)(i) of this section that the testing shall be initiated.

(B) Progress reports shall be submitted to EPA every 6 months, beginning 6 months after the date of notification that the testing shall be initiated, until submission of the final report to EPA.

(4) *Pharmacokinetics*—(i) *Required testing.* (A) Pharmacokinetics testing of DGBE and DGBA will be conducted in rats by the dermal route of administration in accordance with § 795.225 of this chapter, except for the provisions in paragraphs (b) (1)(ii) and (3)(i) of § 795.225.

(B) For the purpose of this section, the following provisions also apply:

(*1*) *Animals.* Adult male and female Sprague Dawley rats shall be used. The rats shall be 7 to 8 weeks old and weigh 180 to 220 grams. Prior to testing, the animals shall be selected at random for each group. Animals showing signs of ill health shall not be used.

(*2*) *Observation of animals—Urinary and fecal excretion.* The quantities of $^{14}C$ excreted in urine and feces by rats dosed as specified in paragraph (b)(2)(iv) of § 795.225 shall be determined at 8, 24, 48, 72, and 96 hours after dosing, and if necessary, daily thereafter until at least 90 percent of the dose has been excreted or until 7 days after dosing (whichever occurs first). Four animals per sex per dose group shall be used for this purpose.

(ii) *Reporting requirements.* (A) The pharmacokinetics tests shall be completed and the final reports submitted to EPA within 12 months of the effective date of the final rule.

(B) A progress report shall be submitted to EPA 6 months from the effective date of the final rule.

(d) *References.* For additional background information the following references should be consulted:

(1) Lamb, J.C. and Chapin, R.E. "Experimental models of male reproductive toxicology." In: "Endocrine Toxicology." Thomas, J.A., Korach, K.S., and McLachlan, J.A., eds. New York, NY: Raven Press. pp. 85-115. (1985).

(2) Clermont, Y. and Perey, B. "Quantitative study of the cell population of the seminiferous tubules in immature rats." *American Journal of Anatomy*. 100:241–267. (1957).

(3) Sadleir, R.M.F.S. "Cycles and seasons." In: "Reproduction in Mammals: 1 Germ Cells and Fertilization." Austin, C.R. and Short, R.V., eds. New York, NY: Cambridge Press. Chapter 4. (1978).

(4) Mattison, D.R. and Thorgiersson, S.S. "Ovarian aryl hydrocarbon hydroxylase activity and primordial oocyte toxicity of polycyclic aromatic hydrocarbons in mice." *Cancer Research*. 39:3471–3475. (1979).

(5) Pederson, T. and Peters, H. "Proposal for classification of oocytes and follicles in the mouse ovary. *Journal of Reproduction and Fertility*. 17:555–557. (1968).

(6) Spencer, P.S., Bischoff, M.C., and Schaumburg, H.H. "Neuropathological methods for the detection of neurotoxic disease." In: "Experimental and Clinical Neurotoxicology." Spencer, P.S. and Schaumburg, H.H., eds. Baltimore, MD: Williams & Wilkins, pp. 743-757. (1980).

(7) Hafez, E.S., ed., "Reproduction and Breeding Techniques for Laboratory Animals." Chapter 10. Philadelphia: Lea & Febiger (1970).

(e) *Effective dates*. (1) The effective date of the final rule shall be April 11, 1988.

(2) The guidelines and other test methods cited in this section are referenced here as they exist on April 11, 1988.

(Information collection requirements have been approved by the Office of Management and Budget under control number 2070-0033)

[53 FR 5950, Feb. 26, 1988, as amended at 54 FR 27357, June 29, 1989]

### § 799.1575 Diethylenetriamine (DETA).

(a) *Identification of chemical test substance*. (1) Diethylenetriamine (CAS No. 111-40-0, also known as DETA) shall be tested in accordance with this part.

(2) Diethylenetriamine of at least 99 percent purity shall be used as the test substances in all tests.

(b) *Persons required to submit study plans, conduct tests and submit data*. All persons who manufacture or process diethylenetriamine from July 8, 1985, to the end of the reimbursement period shall submit letters of intent to test, exemption applications, and study plans and shall conduct tests and submit data as specified in this section, Subpart A of this part and Part 790 of this chapter (Test Rule Development and Exemption Procedures).

(c) *Health effects testing*—(1) *Mutagenic effects—Gene mutation*—(i) *Required testing*. (A) A sex-linked recessive lethal test in *Drosophila melanogaster* shall be conducted with DETA.

(B) A mouse specific locus assay shall be conducted with DETA, if the sex-linked recessive lethal test in *Drosophila melanogaster* conducted pursuant to paragraph (c)(1)(i)(A) of this section produces a positive result.

(ii) *Test standards*. (A) The testing for the sex-linked recessive lethal assay shall be conducted in accordance with the following revised EPA-approved modified study plan (June 19, 1986) originally submitted by the Diethylenetriamine Producers/Importers Alliance (DPIA): "Sex-linked recessive lethal test in *Drosophila melanogaster*," with modifications as approved by EPA on March 9, 1987, and May 21, 1987.

(B) The testing for the mouse visible specific locus assay shall be conducted in accordance with the following revised EPA-approved modified study plan (June 19, 1986) originally submitted by the Diethylenetriamine Producers/Importers Alliance (DPIA): "Mouse specific locus test for visible markers."

(C) These revised EPA-approved modified study plans are available for inspection in EPA's OPTS Reading Room, Rm. NE-G004, 401 M St., SW., Washington, DC 20460.

(iii) *Reporting requirements*. (A) The sex-linked recessive lethal test of DETA in *Drosophila melanogaster* shall be completed and a final report submitted to the Agency within 14 months from the effective date of the final Phase II rule. Two interim progress reports shall be submitted at 6-month intervals, the first of which is due within 6 months of the effective date of the final Phase II rule.

(B) If required pursuant to paragraph (c)(1)(i)(B) of this section, the mouse specific locus test of DETA for visible markers shall be completed and a final report submitted to the Agency within 48 months from the designated date contained in EPA's notification of the test sponsor by certified letter or FEDERAL REGISTER notice that testing should be initiated. Seven interim progress reports shall be submitted at 6-month intervals, the first of which is due within 6 months of EPA's designated date.

(2) *Mutagenic effects—Chromosomal aberrations*—(i) *Required testing.* (A) An *in vitro* cytogenetics test shall be conducted with DETA.

(B) An *in vivo* cytogenetics test shall be conducted with DETA, if the *in vitro* cytogenetics test conducted pursuant to paragraph (c)(2)(i)(A) of this section produces a negative result.

(C) A dominant lethal assay shall be conducted with DETA, if either the *in vitro* cytogenetics test conducted pursuant to paragraph (c)(2)(i)(A) of this section or the *in vivo* cytogenetics test conducted pursuant to paragraph (c)(2)(i)(B) of this section produces a positive result.

(D) A heritable translocation assay shall be conducted with DETA, if the dominant lethal assay conducted pursuant to paragraph (c)(2)(i)(C) of this section produces a positive result.

(ii) *Test standards.* (A) The testing for cytogenetic effects shall be conducted in accordance with the following revised EPA-approved modified study plan (June 19, 1986) originally submitted by the Diethylenetriamine Producers/Importers Alliance (DPIA): "*In vitro* cytogenetics test" and "*In vivo* cytogenetics test," with modifications as approved by EPA on March 9, 1987, and May 21, 1987.

(B) Other testing for cytogenetic effects shall be conducted in accordance with the following revised EPA-approved modified study plans (June 19, 1986) originally submitted by the Diethylenetriamine Producers/Importers Alliance (DPIA): "Dominant lethal assay of diethylenetriamine in CD rats," and "Heritable translocation of diethylenetriamine in CD-1 mice."

(C) These revised EPA-approved modified study plans are available for inspection in EPA's OPTS Reading Room, Rm. NE-G004, 401 M St., SW., Washington, DC 20460.

(iii) *Reporting requirements.* (A) The *in vitro* cytogenetics testing of DETA shall be completed and a final report submitted to the Agency within 6 months of the effective date of the final Phase II rule.

(B) If required pursuant to paragraph (c)(2)(i)(B) of this section, the *in vivo* cytogenetics testing of DETA shall be completed and final report submitted to the Agency within 14 months of the effective date of the final Phase II rule. One interim progress report shall be submitted within 12 months of the final rule's effective date.

(C) If required pursuant to paragraph (c)(2)(i)(C) of this section, the dominant lethal testing of DETA shall be completed and a final report submitted to the Agency within 20 months of the effective date of the final Phase II rule.

(D) If required pursuant to paragraph (c)(2)(i)(D) of this section, the heritable translocation testing of DETA shall be completed and a final report submitted to the Agency within 18 months of the designated date contained in EPA's notification of the test sponsor by certified letter or FEDERAL REGISTER notice that testing should be initiated. Two interim progress reports shall be submitted at 6-month intervals, the first of which is due within 6 months of EPA's designated date.

(3) *Subchronic effects*—(i) *Required testing.* A ninety-day oral subchronic toxicity test shall be conducted with DETA in at least one mammalian species.

(ii) *Test Standard.* The testing shall be conducted in accordance with the following revised EPA-approved modified study plans (June 19, 1986) originally submitted by the Diethylenetriamine Producers/Importers Alliance (DPIA): "Ninety-Day (subchronic) dietary toxicity study with diethylenetriamine in albino rats," with modifications approved by EPA on March 9, 1987, and May 21, 1987. This revised EPA-approved modified study plan is available for inspection in EPA's OPTS Reading Room, Rm. NE-G004, 401 M St., SW., Washington, DC 20460.

(iii) *Reporting requirements.* The testing shall be completed and a final report submitted to the Agency within 15 months of the effective date of the final Phase II rule. Two interim progress reports shall be submitted at 6-month intervals, the first of which is due within 6 months of the effective date of the final Phase II rule.

(4) *Dermal absorption*—(i) *Required testing.* A dermal absorption test shall be conducted with DETA in the same mammalian species used for the oral subchronic (90-day) test conducted pursuant to paragraph (c)(3)(i) of this section.

(ii) *Test standard.* The testing shall be conducted in accordance with the following revised EPA-approved modified study plan (June 19, 1986) originally submitted by the Diethylenetriamine Producers/Importers Alliance (DPIA): "Dermal absorption," with modifications approved by EPA on March 9, 1987, May 21, 1987, and December 16, 1987. This revised EPA-approved modified study plan is available for inspection in EPA's OPTS Reading Room, Rm. NE-G004, 401 M St., SW., Washington, DC 20460.

(iii) *Reporting requirements.* The testing shall be completed and the final report submitted to the Agency within 20 months of the effective date of the final Phase II rule. Three interim progress reports shall be submitted at 6-month intervals, the first of which is due within 6 months of the effective date of the final Phase II rule.

(d) *Chemical fate testing*—(1) *Required testing.* Testing to assess *N*-nitrosamine formation, resulting from aerobic biological and/or chemical transformation, shall be conducted with DETA using environmental samples of lake water, sewage, and soil.

(2) *Test standard.* The testing shall be conducted in accordance with the following revised EPA-approved modified study plan (June 19, 1986) originally submitted by the Diethylenetriamine Producers/Importers Alliance (DPIA): "Chemical fate," with modifications approved by EPA on March 9, 1987, May 21, 1987, July 9, 1987, and December 16, 1987. This revised EPA-approved modified study plan is available for inspection in EPA's OPTS Reading Room, Rm. NE-G004, 401 M St., SW., Washington, DC 20460.

(3) *Reporting requirements.* The testing shall be completed and a final report submitted to the Agency within 18 months of the effective date of the final Phase II rule. Two interim progress reports shall be submitted at 6-month intervals, the first of which is due within 6 months of the effective date of the final Phase II rule.

(e) *Modifications.* Persons subject to this section are not subject to the requirements of § 790.50(a)(2)(ii) of this chapter.

(f) *Effective date.* The effective date of the final Phase II rule for diethylenetriamine is March 19, 1987.

(Approved by the Office of Management and Budget under control number 2070-0033)

[50 FR 21412, May 23, 1985; 50 FR 33543, Aug. 20, 1985; 51 FR 3468, Jan. 28, 1986; 51 FR 4736, Feb. 7, 1986; 52 FR 3238, Feb. 3, 1987; 54 FR 27356, June 29, 1989]

### § 799.1645 2-Ethylhexanol.

(a) *Identification of test substance.* (1) 2-Ethylhexanol (CAS No. 104-76-7) shall be tested in accordance with this section.

(2) 2-Ethylhexanol of at least 99.0-percent purity shall be used as the test substance.

(b) *Persons required to submit study plans, conduct tests, and submit data.* All persons who manufacture or process, or intend to manufacture or process 2-ethylhexanol, other than as an impurity, from the effective date of this final rule to the end of the reimbursement period shall submit letters of intent to conduct testing, submit study plans, conduct tests, and submit data or exemption applications as specified in this section, Subpart A of this part, and Parts 790 and 792 of this chapter for single-phase rulemaking.

(c) *Health effects*—(1) *Oncogenic effects*— (i) *Required testing.* (A) Oncogenicity tests shall be conducted in Fisher 344 rats and B6C3Fl mice by the oral route with 2-ethylhexanol in accordance with § 798.3300 of this chapter, except for the provisions in § 798.3300(b)(6).

(B) For the purpose of this section, the following provisions also apply to the oncogenicity tests: (*1*) *Administration of the test substance.* 2-Ethylhexanol shall be administered either by microencapsulation before adding it to the diet or by gavage.

(*2*) [Reserved]

(ii) *Reporting requirements.* (A) The study plan for the oncogenicity test shall be submitted at least 45 days before the initiation of testing.

(B) The oncogenicity testing shall be completed and final report submitted to the Agency within 53 months of the effective date of this final rule if 2-ethylhexanol is administered by gavage or within 56 months of the effective date of this final rule if administered by microencapsulation.

(C) Interim progress reports shall be submitted to EPA at 6-month intervals beginning 6 months after the effective date of the final rule, until the final report is submitted to EPA.

(2) [Reserved]

(d) *Effective date.* The effective date of this final rule requiring oncogenicity testing of 2-ethylhexanol is September 16, 1987.

(Information collection requirements are approved by the Office of Management and Budget under control number 2070-0033)

[52 FR 28704, Aug. 3, 1987]

### § 799.1650 2-Ethylhexanoic acid.

(a) *Identification of test substance.* (1) 2-Ethylhexanoic acid (CAS No. 149-57-5) (hereinafter "EHA") shall be tested in accordance with this section.

(2) EHA of at least 99-percent purity shall be used as the test substance.

(b) *Persons required to submit study plans, conduct tests, and submit data.* All persons who manufacture or process EHA other than as an impurity from the effective date of this section, December 20, 1986, to the end of the reimbursement period shall submit an exemption application, or shall submit a letter of intent to conduct testing, study plans, conduct tests, and submit data as specified in this section, Subpart A of this part, and Parts 790 and 792 of this chapter. The end of the reimbursement period shall be 5 years after the submission of the last final report required under this test rule.

(c) *Health effects testing*—(1) *Pharmacokinetics*—(i) *Required testing.* Metabolism studies of the oral and dermal routes of exposure shall be conducted with EHA using Fischer 344 rats in accordance with the test standard specified in § 795.223 of this chapter.

(ii) *Reporting requirements.* (A) Study plans shall be provided to the Agency at least 45 days prior to initiating testing.

(B) An interim progress report shall be provided to the Agency 6 months after the effective date of the final test rule.

(C) The final report of results shall be submitted to the Agency no later than 1 year from the effective date of the final test rule.

(2) *Subchronic toxicity*—(i) *Required testing.* Subchronic toxicity tests shall be conducted with EHA using Fischer 344 rats and B6C3F1 mice in accordance with the test standard specified in § 795.260 of this chapter.

(ii) *Reporting requirements.* (A) Study plans shall be provided to the Agency at least 45 days prior to initiating testing.

(B) Interim progress reports shall be provided to the Agency 6 months and 12 months after the effective date of the final test rule.

(C) The final report of results shall be submitted to the Agency no later than 18 months from the effective date of the final test rule.

(3) *Administration of test substance.* Dosing for the testing required under paragraphs (c) (1) and (2) of this section shall be by the oral route for both tests, and as specified in § 795.223(c)(2)(ii)(C) and § 795.260(d)(7) of this chapter.

(4) *Development toxicity*—(i) *Required testing.* Developmental toxicity tests shall be conducted with EHA using one rodent and one nonrodent mammalian species in accordance with the OECD guideline entitled "Teratogenicity", No. 414, adopted May 12, 1981. The OECD guideline is available in OECD Publication No. ISBN 92-64-12221-4 and is sold by the OECD Publication and Information Center, Room Number 1207, 1750 Pennsylvania Avenue, NW., Washington, DC. Copies of this document may be in-

spected at the Office of the Federal Register, 1100 L Street, NW., Room 8401, Washington, DC, or the OPTS Reading Room (docket No. OPTS-42065), Room N.E.-G004, Environmental Protection Agency, 401 M Street, SW., Washington, DC. This incorporation by reference was approved by the Director of the Federal Register in accordance with 5 U.S.C. 552(a) and 1 CFR Part 51. These materials are incorporated as they exist on the effective date of this rule; a notice of any change will be published in the FEDERAL REGISTER.

(ii) *Reporting requirements.* (A) Study plans shall be provided to the Agency at least 45 days prior to initiating testing.

(B) Interim progress reports shall be provided to the Agency 6 months and 12 months after the effective date of the final test rule.

(C) The final report of results shall be submitted to the Agency no later than 18 months from the effective date of the final test rule.

(Information collection requirements are approved by the Office of Management and Budget under control number 2070-0033)

[51 FR 40330, Nov. 6, 1986, as amended at 52 FR 24158, June 29, 1987; 52 FR 32240, Aug. 26, 1987]

## § 799.1700 Fluoroalkenes.

(a) *Identification of test substances.* (1) Vinyl fluoride (VF; CAS No. 75-02-5), vinylidene fluoride (VDF; CAS No. 75-38-7), tetrafluoroethene (TFE; CAS No. 116-14-3), and hexafluoropropene (HFP; CAS No. 116-15-4) shall be tested in accordance with this section.

(2) VF, VDF, TFE, and HFP of at least 99 percent purity shall be used as the test substances.

(b) *Persons required to submit study plans, conduct tests and submit data.* All persons who manufacture VF, VDF, TFE, or HFP, other than as an impurity, from July 22, 1987 to the end of the reimbursement period shall submit letters of intent to conduct testing or exemption applications, submit study plans, conduct tests in accordance with the TSCA Good Laboratory Practice Standards (40 CFR Part 792), and submit data as specified in this section, Subpart A of this part, and Part 790 of this chapter for single-phase rulemaking, for the substances they manufacture.

(c) *Health effects testing*—(1) *Mutagenic effects—Gene mutation*—(i) *Required testing.* (A) (*1*) A detection of gene mutations in somatic cells in culture assay shall be conducted with TFE and HFP in accordance with § 798.5300 of this chapter except for the provisions in paragraphs (c), (d)(3)(i), (4), (5) and (6) and (e).

(*2*) For the purposes of this section, the following provisions also apply:

(*i*) *Reference substances.* No reference substance is required.

(*ii*) *Test method—Type of cells used in the assay.* Mutation induction at the HPRT locus shall be measured in Chinese hamster ovary (CHO) cells. Cells shall be checked for Mycoplasma contamination and may also be checked for karyotype stability.

(*iii*) *Test method—Metabolic activation.* Cells shall be exposed to the test substance only in the presence of a metabolic activation system for TFE, and in both the presence and absence of a metabolic activation system for HFP. The metabolic activation system shall be derived from the post-mitochondrial fraction (S-9) of livers from rats pretreated with Aroclor 1254.

(*iv*) *Test method—Control groups.* Positive and negative controls shall be included in each experiment. In assays with metabolic activation, the positive control substance shall be known to require such activation. Nitrogen shall serve as the negative control and diluting gas.

(*v*) *Test method—Test chemicals.* The test should be designed to have a predetermined sensitivity and power. The number of cells, cultures, and concentrations of test substance used should reflect these defined parameters. The number of cells per culture is based on the expected background mutant frequency; a general guide is to use a number which is 10 times the inverse of this frequency. Several concentrations (usually at least four) of the test substance shall be used. These shall yield a concentration-related toxic effect. The highest concentration shall produce a low level of survival (approximately 10 percent), and the survival in the lowest concentration shall approximate that of the negative control. Cytotoxicity shall be determined after treatment with the test substance both in the presence and in the absence of the metabolic activation system.

(*vi*) *Test performance.* Cells in treatment medium with and without metabolic activation shall be exposed to varying concentrations of test gas-air mixtures by flushing treatment flasks (or chambers) with 10 volumes of test gas-air mixture at a rate of 500 mL/min or that rate which will allow complete flushing within 1 minute. In the case of a test chamber volume of 1.67 L, a flow rate of 10 L/min is appropriate. Each flask shall be closed with a cap with a rubber septum. Headspace samples shall be taken at the beginning and end of the exposure period and analyzed to determine the amount of test gas in each flask. Flasks shall be incubated on a rocker panel at 37 °C for 5 hours for tests with metabolic activation. For the non-activated portion of the test, the incubation time shall be 18 to 19 hours at 37 °C. At the end of the exposure period, cells treated with metabolic activation shall be washed and incubated in culture medium for 21 to 26 hours prior to subculturing the viability and expression of mutant phenotype. Cells treated without metabolic activation shall be washed and subcultured immediately to determine viability and to allow for expression of mutant phenotype. Appropriate subculture schedules (generally twice during the expression period) shall be used. At the end of the expression period, which shall be sufficient to allow near optimal phenotypic expression of induced mutants (generally 7 days for this cell system), cells shall be grown in medium with and without selective agent for determination of numbers of mutants and cloning efficiency, respectively. This last growth period is generally 7 days at 37 °C. Results of this test shall be confirmed in an independent experiment.

(B)(*1*) A sex-linked recessive lethal test in *Drosophila melanogaster* shall be conducted with VDF and VF in accordance with § 798.5275 of this chapter except for the provisions in paragraph (d)(5). This test shall also be performed with TFE or HFP if the somatic cells in culture assay conducted pursuant to paragraph (c)(1)(i)(A) of this section produces a positive result.

(*2*) For the purposes of this section the following provisions also apply:

(*i*) *Test chemicals.* It is sufficient to test a single dose of the test substance. This dose shall be the maximum tolerated dose or that which produces some indication of toxicity. Exposure shall be by inhalation.

(*ii*) [Reserved]

(C)(*1*) A mouse specific locus assay shall be conducted with VF, VDF, TFE, and HFP in accordance with § 798.5200 of this chapter, except for the provisions of paragraph (d)(5), for whichever of these substances produces a positive result in the sex-linked recessive lethal test in *Drosophila melanogaster* conducted pursuant to paragraph (c)(1)(i)(B) of this section if, after a public program review, EPA issues a Federal Register notice or sends a certified letter to the test sponsor specifying that the testing shall be initiated.

(*2*) For the purposes of this section, the following provisions also apply:

(*i*) *Test chemicals.* A minimum of two dose levels shall be tested. The highest dose tested shall be the highest dose tolerated without toxic effects, provided that any temporary sterility induced due to elimination of spermatagonia is of only moderate duration, as determined by a return of males to fertility within 80 days after treatment, or shall be the highest dose attainable. Animals shall be exposed to the test substance by inhalation. Exposure shall be for 6 hours a day. Duration of exposure shall be dependent upon accumulated total dose desired for each group.

(*ii*) [Reserved]

(ii) *Reporting requirements.* (A) Mutagenic effects-gene mutation tests shall be completed and the final results submitted to EPA as follows: Somatic cells in culture assay, by May 16, 1988; *Drosophila* sex-linked recessive lethal, by August 15, 1988 (for VF and VDF), and within 15 months (for TFE and HFP) after the effective date of the final rule; mouse visible specific locus assay, within 51 months after the date of EPA's notification of the test sponsor by certified letter or Fed-

ERAL REGISTER notice that testing shall be initiated.

(B) Progress reports shall be submitted to the Agency every 6 months beginning 6 months after the effective date of the final rule or receipt of notice that testing shall be initiated.

(2) *Mutagenic effects—Chromosomal aberrations*—(i) *Required testing.* (A) (*1*) A mouse micronucleus cytogenetics test shall be conducted with VDF and TFE in accordance with § 798.5395 of this chapter except for the provisions in paragraphs (d)(5) (i), (ii), and (iii).

(*2*) For the purposes of this section, the following provisions also apply:

(*i*) *Test method—Vehicle.* No vehicle is required.

(*ii*) *Test method—Dose levels.* Three dose levels shall be used. The highest dose tested shall be the maximum tolerated dose, that dose producing some indication of cytotoxicity (e.g., a change in the ratio of polychromatic to normochromatic erythrocytes, or the highest dose attainable).

(*iii*) *Test method—route of administration.* Animals shall be exposed by inhalation with a single 6-hour exposure, with three sampling times between 29 and 72 hours.

(B)(*1*) For each respective test substance, a dominant lethal assay shall be conducted with VF and HFP in accordance with § 798.5450 of this chapter except for the provisions in paragraphs (d)(2)(i), (4)(i), (5) and (e). This test shall also be performed with TFE or VDF if the mouse micronucleus cytogenetics test conducted pursuant to paragraph (c)(2)(i)(A) of this section produces a positive result.

(*2*) For the purposes of this section, the following provisions also apply:

(*i*) *Test method—Description.* For this assay, the test substance shall be administered by inhalation for 5 consecutive days for 6 hours per day.

(*ii*) *Test method—Concurrent controls.* Concurrent positive and negative (vehicle) controls shall be included in each experiment.

(*iii*) *Test method—Test chemicals.* Exposure shall be by inhalation for 5 consecutive days for 6 hours per day. Three dose levels shall be used. The highest dose shall produce signs of toxicity (e.g., slightly reduced fertility) or shall be the highest attainable.

(*iv*) *Test performance.* Individual males shall be mated sequentially to 1 or 2 virgin females. Females shall be left with the males for at least the duration of one estrus cycle or alternatively until mating has occurred as determined by the presence of sperm in the vagina or by the presence of a vaginal plug. In any event, females shall be left with the males for no longer than 7 days. The number of matings following treatment shall ensure that germ cell maturation is adequately covered. Mating shall continue for at least 6 weeks. Females shall be sacrificed in the second half of pregnancy, and uterine contents shall be examined to determine the number of implants and live and dead embryos. The examination of ovaries to determine the number of corpora lutea is left to the discretion of the investigator.

(C)(*1*) A heritable translocation assay shall be conducted with VF, VDF, TFE, or HFP in accordance with § 798.5460 of this chapter except for the provisions of paragraphs (d)(3)(i), (5), and (e)(1), if the dominant lethal assay conducted for that substance pursuant to paragraph (c)(2)(i)(B) of this section produces a positive result and if, after a public program review, EPA issues a FEDERAL REGISTER notice or sends a certified letter to the test sponsor specifying that the testing shall be initiated.

(*2*) For the purposes of this section, the following provisions also apply:

(*i*) *Test method—Animal selection.* The mouse shall be used as the test species.

(*ii*) *Test method.* No vehicle is required. At least two dose levels shall be used. The highest dose level shall result in toxic effects (which shall not produce an incidence of fatalities which would preclude a meaningful evaluation) or shall be the highest dose attainable. Animals shall be exposed by inhalation.

(*iii*) *Test performance—Treatment and mating.* The animals shall be dosed with the test substance 6 hours per day, 7 days per week over a period of 35 days. After treatment, each male shall be caged with 2 untreated females for a period of 1 week. At the end of 1 week, females shall be separated from males and caged individually. When females give birth, the date of birth, litter size and sex of progeny shall be recorded. All male progeny shall be weaned and all female progeny shall be discarded.

(ii) *Reporting requirements.* (A) Mutagenic effects-chromosomal aberration testing shall be completed and final results submitted to EPA after the effective date of the rule as follows: mouse micronucleus cytogenetics for VDF by November 22, 1988, and for TFE within 10 months after the effective date of the final rule; dominant lethal assay for VF and HFP by October 22, 1988, and for VDF and TFE within 19 months after the effective date of the rule; heritable translocation assay, within 25 months after the date of EPA's notification of the test sponsor by certified letter or FEDERAL REGISTER notice that testing shall be initiated.

(B) Progress reports shall be submitted to the Agency every 6 months beginning 6 months after the effective date of the final rule or receipt of notice that testing shall be initiated.

(3) *Subchronic toxicity*—(i) *Required Testing.* (A) An inhalation subchronic toxicity test shall be conducted with HFP in accordance with the TSCA Test Guideline specified in § 798.2450 of this chapter except for the provisions in paragraphs (d)(5), (10)(v), and (e)(3)(iv)(D).

(B) For the purpose of this section the following provisions also apply:

(*1*) *Test procedures—Exposure conditions.* The animals shall be exposed to the test substance 6 hours per day, 5 days per week for 90 days.

(*2*) *Test procedures—Observation of animals.* Animals shall be weighted weekly, and food and water consumption shall also be measured weekly.

(*3*) *Test report—Individual animal data.* Food and water consumption data shall be reported.

(ii) *Reporting requirements.* (A) The required subchronic toxicity test shall be completed and final results submitted to the Agency within 18 months after the effective date of the final rule.

(B) Progress reports shall be submitted to the Agency every 6 months beginning 6 months after the effective date of the final rule.

(4) *Oncogenicity*—(i) *Required testing.* Oncogenicity tests shall be conducted in both rats and mice by inhalation with VF and in mice with VDF in accordance with § 798.3300 of this chapter. Oncogenicity tests shall also be conducted by inhalation in both rats and mice with TFE in accordance with § 798.3300 of this chapter if TFE yields a positive result in any one of the following mutagenicity tests: The mouse micronucleus cytogenetics assay conducted pursuant to paragraph (c)(2)(i)(A) of this section, the mammalian cells in culture assay conducted pursuant to paragraph (c)(1)(i)(A) of this section or the sex-linked recessive lethal assay in *Drosophila melanogaster* conducted pursuant to paragraph (c)(1)(i)(B) of this section if, after public program review, EPA issues a FEDERAL REGISTER notice or sends a certified letter to the test sponsor specifying that testing shall be initiated.

Oncogenicity tests shall also be conducted by inhalation in both rats and mice with TFE in accordance with § 798.3300 of this chapter if TFE yields a positive test result in any one of the following mutagenicity tests: The *in vitro* cytogenetics assay conducted pursuant to paragraph (c)(2)(i)(A) of this section, the mouse micronucleus cytogenetics assay conducted pursuant to paragraph (c)(2)(i)(B) of this section, the mammalian cells in culture assay conducted pursuant to paragraph (c)(1)(i)(A) of this section or the sex-linked recessive lethal assay in *Drosophila melanogaster* conducted pursuant to paragraph (c)(1)(i)(B) of this section if, after a public program review, EPA issues a FEDERAL REGISTER notice or sends a certified letter to the test sponsor specifying that the testing shall be initiated. Criteria for positive test results are established in 40 CFR 798.5375, 798.5385, 798.5300 and 798.5275 of this chapter, respectively.

(ii) *Reporting requirements.* (A) The oncogenicity testing shall be completed and the final results submitted to the Agency within 56 months after the effective date of the final rule for VF and VDF. For TFE and HFP, the oncogenicity testing shall be completed and final results submitted to the

Agency within 56 months after the date of EPA's notification of the test sponsor by certified letter or FEDERAL REGISTER notice that testing shall be initiated.

(B) Progress reports shall be submitted every 6 months beginning 6 months after the effective date of the final rule for VF and VDF and beginning 6 months after notification by certified letter or FEDERAL REGISTER notice that testing is to begin for TFE and HFP.

(d) *Effective date.* The effective date of this final rule is July 22, 1987.

(Information collection requirements have been approved by the Office of Management and Budget under control number 2070-0033)

[52 FR 21530, June 8, 1987, as amended at 52 FR 43762, Nov. 16, 1987; 54 FR 27357, June 29, 1989]

### § 799.2155 Commercial hexane.

(a) *Identification of test substance.* (1) "Commercial hexane," for purposes of this section, is a product obtained from crude oil, natural gas liquids, or petroleum refinery processing in accordance with the American Society for Testing and Materials Designation D 1836-83 (ASTM D 1836), consists primarily of six-carbon alkanes or cycloalkanes, and contains at least 40 liquid volume percent *n*-hexane (CAS No. 110-54-3) and at least 5 liquid volume percent methylcyclopentane (MCP; CAS No. 96-37-7). ASTM D 1836, formally entitled "Standard Specification for Commercial Hexanes," is published in *1986 Annual Book of ASTM Standards: Petroleum Products and Lubricants,* ASTM D 1836-83, pp. 966-967, 1986, is incorporated by reference, and is available for public inspection at the Office of the Federal Register, Room. 8301, 1100 L Street NW., Washington, DC. This incorporation by reference was approved by the Director of the Office of the Federal Register in accordance with 5 U.S.C. 522(a) and 1 CFR Part 51. This material is incorporated as it exists on the date of approval, and a notice of any change in this material will be published in the FEDERAL REGISTER. Copies of the incorporated material may be obtained from the Document Control Officer (TS-793), Office of Toxic Substances, EPA, Room. NE-G004, 401 M Street SW., Washington, DC 20460.

(2) The commercial hexane test substance, for purposes of this section, is a product which conforms to the specifications of ASTM D1836 and contains at least 40 liquid volume percent but no more than 55 liquid volume percent *n*-hexane and no less than 10 liquid volume percent MCP.

(b) *Persons required to submit study plans, conduct tests, and submit data.* All persons who manufacture (including import) or process or intend to manufacture or process commercial hexane, as defined in paragraph (a)(1) of this section and other than as an impurity, from the effective date of the final rule to the end of the reimbursement period shall submit letters of intent to conduct testing, submit study plans, conduct tests in accordance with Part 792 of this chapter, and submit data, or submit exemption applications, as specified in this section, Subpart A of this part, and Part 790 of this chapter for single-phase rulemaking. Persons who manufacture commercial hexane as a byproduct are covered by the requirements of this section. Notwithstanding § 790.50(a)(1) of this chapter, persons who notify EPA of their intent to conduct neurotoxicity testing in compliance with paragraph (c)(7) of this section may submit study plans for those tests less than 45 days before beginning testing provided that EPA receives the study plans before this testing begins.

(c) *Health effects testing—(1) Subchronic inhalation toxicity—(i) Required testing.* (A) A subchronic inhalation toxicity test shall be conducted with commercial hexane in accordance with § 798.2450 of this chapter except for the provisions in paragraphs (d)(4)(ii) and (5) of § 798.2450.

(B) For the purposes of this section, the following provisions also apply:

(*1*) *High dose level.* The highest concentration should result in toxic effects but neither produce an incidence of fatalities which would prevent a meaningful evaluation nor exceed the lower explosive limit of commercial hexane.

(*2*) *Exposure conditions.* Animals shall be dosed for 6 hours/day, 5 days/week for 90 days.

(ii) *Reporting requirements.* (A) The subchronic inhalation toxicity test shall be completed and the final report submitted to EPA within 15 months of the effective date of the final rule.

(B) Interim progress reports shall be submitted to EPA for the subchronic inhalation toxcity test at 6-month intervals beginning 6 months after the effective date of the final rule, until the final report is submitted to EPA.

(2) *Oncogenicity*—(i) *Required testing.* (A) An oncogenicity test shall be conducted with commercial hexane in accordance with § 798.3300 of this chapter except for the provisions in paragraphs (b)(3)(ii) and (6) of § 798.3300.

(B) For the purposes of this section, the following provisions also apply:

(*1*) *High dose level.* The high dose level should elicit signs of minimal toxicity without substantially altering the normal life span and should not exceed the lower explosive limit of commercial hexane.

(*2*) *Administration of test substance.* Animals shall be exposed to commercial hexane by inhalation.

(ii) *Reporting requirements.* (A) The oncogenicity test shall be completed and the final report submitted to EPA within 53 months of the effective date of the final rule.

(B) Interim progress reports shall be submitted to EPA for the oncogenicity test at 6-month intervals beginning 6 months after the effective date of the final rule, until the final report is submitted to EPA.

(3) *Reproduction and fertility effects*—(i) *Required testing.* (A) A reproduction and fertility effects test shall be conducted with commercial hexane in accordance with § 798.4700 of this chapter except for the provisions in paragraphs (c)(3)(ii) and (5) of § 798.4700.

(B) For the purposes of this section, the following provisions also apply:

(*1*) *High dose level.* The highest dose level should induce toxicity but not high levels of mortality in the parental (P) animals. In addition, the highest dose level should not exceed the lower explosive limit of commercial hexane.

(*2*) *Administration of test substance.* Animals shall be exposed to commercial hexane by inhalation.

(ii) *Reporting requirements.* (A) The reproduction and fertility effects test shall be completed and the final report submitted to EPA within 29 months of the effective date of the final rule.

(B) Interim progress reports shall be submitted to EPA for the reproduction and fertility effects test at 6-month intervals beginning 6 months after the effective date of the final rule, until the final report is submitted to EPA.

(4) *Inhalation developmental toxicity*—(i) *Required testing.* (A) An inhalation dèvelopmental toxicity test shall be conducted with commercial hexane in accordance with § 795.4350 of this chapter except for the provisions in paragraph (e)(3)(iv) of § 798.4350.

(B) For the purposes of this section, the following provisions also apply:

(*1*) *High dose level.* Unless limited by the physical/chemical nature or biological properties of the test substance, the highest concentration level shall induce some overt maternal toxicity such as reduced body weight or body weight gain, but not more than 10 percent maternal deaths. In addition, the highest dose level should not exceed the lower explosive limit of commercial hexane.

(*2*) [Reserved]

(ii) *Reporting requirements.* (A) The inhalation developmental toxicity test shall be completed and the final report submitted to EPA within 12 months of the effective date of the final rule.

(B) Interim progress reports shall be submitted to EPA for the inhalation developmental toxicity test at 6-month intervals beginning 6 months after the effective date of the final rule, until the final report is submitted to EPA.

(5) *Mutagenic effects—gene mutations*—(i) *Required testing.* (A)(*1*) A *Salmonella typhimurium* reverse mutation assay shall be conducted with commercial hexane in accordance with § 798.5265 of this chapter except for the provisions in paragraphs (d)(4) and (e) of § 798.5265.

(*2*) For the purposes of this section, the following provisions also apply:

(*i*) *Metabolic activation.* Bacteria shall be exposed to commercial hexane both in the presence and absence of an appropriate metabolic activation system.

(*ii*) *Test performance.* The assay shall be performed using the desiccator method described as follows: The agar overlay plates shall be placed uncovered in a 9-liter desiccator. A volume of the liquid test substance shall be added to the glass Petri dish suspended beneath the porcelain shelf of the desiccator. The highest exposure concentration should not result in a vapor concentration which exceeds the lower explosive limit of commerical hexane. A magnetic stirring bar to serve as a fan to assure rapid and even distribution of the vapor shall be placed on the bottom of the inside of the desiccator. The desiccator shall be placed on a magnetic stirrer within a 37°C room or chamber for 7 to 10 hours. The plates shall then be removed, their lids replaced, followed by incubation for an additional 40 hours at 37°C before counting. An appropriate selective medium with an adequate overlay agar shall be used. All plating should be done in at least triplicate.

(B)(*1*) A gene mutation test in mammalian cells shall be conducted with commercial hexane in accordance with § 798.5300 of this chapter except for the provisions in paragraphs (d)(3)(ii) and (4) of § 798.5300 if the results from the *Salmonella typhimurium* test conducted pursuant to paragraph (c)(5)(i)(A) of this section are negative.

(*2*) For the purposes of this section, the following provisions also apply:

(*i*) *Cell growth and maintenance.* Treatment flasks shall be incubated on a rocker panel to insure maximum contact between the cells and the test agent. Incubation shall be at 37°C for 18 hours for experiments without metabolic activation and for 5 hours for experiments with activation. Each flask shall be closed with a cap with a rubber septum. Headspace samples shall be taken at the beginning and the end of exposure period and analyzed to determine the amount of test substance in each flask. The vapor concentration should not exceed the lower explosive limit of commercial hexane.

(*ii*) [Reserved]

(C)(*1*) A sex-linked recessive lethal test in *Drosophila melanogaster* shall be conducted with commercial hexane in accordance with § 798.5275 of this chapter except for the provisions in paragraphs (d)(5) (ii) and (iii) of § 798.5275, unless the results of both the *Salmonella typhimurium* test conducted pursuant to paragraph (c)(5)(i)(A) of this section and the mammalian cells in the culture gene mutation test conducted pursuant to paragraph (c)(5)(i)(B) of this section, if required, are negative.

(*2*) For the purposes of this section, the following provisions also apply:

(*i*) *Dose levels.* For the initial assessment of mutagenicity, it is sufficient to test a single dose of the test substance for screening purposes. This dose should be the maximum tolerated dose, or that which produces some indication of toxicity or shall be the highest dose attainable and should not exceed the lower explosive limit of commercial hexane. For dose-response purposes, at least three additional dose levels should be used.

(*ii*) *Route of administration.* The route of administration shall be by exposure to commercial hexane vapors.

(D) [Reserved]

(ii) *Reporting requirements.* (A) The gene mutation tests shall be completed and final reports submitted to EPA as follows:

(*1*) The *Salmonella typhimurium* reverse mutation assay within 8 months of the effective date of the final rule.

(*2*) The gene mutation in mammalian cells assay within 17 months of the effective date of the final rule.

(*3*) The sex-linked recessive-lethal test in *Drosophila melanogaster* within 24 months of the effective date of the final rule.

(*4*) [Reserved]

(B) Interim progress reports for each test shall be submitted to EPA for the gene mutation in mammalian cells assay and *Drosophila* sex-linked recessive lethal test at 6-month intervals beginning 6 months after the effective date of the final rule, until the applicable final report is submitted to EPA.

(C) [Reserved]

(6) *Mutagenic effects—chromosomal aberrations*—(i) *Required testing.* (A)(*1*) An *in vitro* cytogenetics test shall be conducted with commercial hexane in accordance with § 798.5375 of this chapter except for the provisions in paragraph (e)(3) of § 798.5375.

(*2*) For the purposes of this section, the following provisions also apply:

(*i*) *Treatment with test substance.* The test shall be performed using flasks flushed with commercial hexane vapors, then closed with a cap with a rubber septum. The highest exposure concentration should not result in a vapor concentration which exceeds the lower explosive limit of commercial hexane.

(*ii*) [Reserved]

(B)(*1*) An *in vivo* cytogenetics test shall be conducted with commercial hexane in accordance with § 798.5385 of this chapter except for the provisions in paragraphs (d)(5) (ii), (iii) and (iv) of § 798.5385, if the *in vitro* test conducted pursuant to paragraph (c)(6)(i)(A) of this section is negative.

(*2*) For the purposes of this section, the following provisions also apply:

(*i*) *Dose levels.* For an initial assessment, one dose level of the test substance may be used, the dose being the maximum tolerated dose (to a maximum of 5,000 mg/kg), or that producing some indication of cytotoxicity (e.g., partial inhibition of mitosis), or shall be the highest dose attainable (to a maximum of 5,000 mg/kg) and should not exceed the lower explosive limit of commercial hexane. Additional dose levels may be used. For determination of dose-response, at least three dose levels should be used.

(*ii*) *Route of administration.* Animals shall be exposed to commercial hexane by inhalation.

(*iii*) *Treatment schedule.* The duration of exposure shall be for 6 hours per day for 5 consecutive days.

(C)(*1*) A dominant lethal assay shall be conducted with commercial hexane in accordance with § 798.5450 of this chapter except for the provisions in paragraphs (d)(5) (ii) and (iii) of § 798.5450, unless both the *in vitro* and *in vivo* cytogenetics tests conducted pursuant to paragraphs (c)(6)(i) (A) and (B) of this section are negative.

(*2*) For the purposes of this section, the following provisions also apply:

(*i*) *Dose levels.* Normally, three dose levels shall be used. The highest dose shall produce signs of toxicity (e.g., slightly reduced fertility and slightly reduced body weight). The highest dose should not exceed the lower explosive limit of commercial hexane. However, in an initial assessment of dominant lethality, a single high dose may be sufficient. Nontoxic substances shall be tested at 5 g/kg or, if this is not practicable, then at the highest dose attainable.

(*ii*) *Route of administration.* Animals shall be exposed to commercial hexane by inhalation.

(*iii*) *Treatment schedule.* The duration of exposure shall be for 6 hours per day for 5 consecutive days.

(D)(*1*) A heritable translocation test shall be conducted with commercial hexane in accordance with § 798.5460 of this chapter except for the provisions in paragraphs (d)(5) (ii) and (iii) of § 798.5460, if the results of the dominant lethal assay conducted pursuant to paragraph (c)(6)(i)(C) of this section are positive and if, after a public program review, EPA issues a FEDERAL REGISTER notice or sends a certified letter to the test sponsor specifying that the testing shall be initiated.

(*2*) For the purposes of this section, the following provisions also apply:

(*i*) *Dose levels.* At least two dose levels shall be used. The highest dose level shall result in toxic effects (which shall not produce an incidence of fatalities which would prevent a meaningful evaluation) or shall be the highest dose attainable or 5 g/kg body weight and should not exceed the lower explosive limit of commercial hexane.

(*ii*) *Route of administration.* Animals shall be exposed to commercial hexane by inhalation.

(*iii*) *Reporting requirements.* (A) The chromosomal aberration tests shall be completed and the final reports submitted to EPA as follows:

(*1*) The *in vitro* cytogenetics test within 9 months of the effective date of the final rule.

(*2*) The *in vivo* cytogenetics test within 19 months of the effective date of the final rule.

(*3*) The dominant lethal assay within 28 months of the effective date of the final rule.

(*4*) The heritable translocation test within 25 months of the date of EPA's notification of the test sponsor by certified letter or FEDERAL REGISTER notice that testing shall be initiated.

(B) Interim progress reports for each test shall be submitted to EPA for the *in vivo* cytogenetics and the dominant lethal assays at 6-month intervals beginning 6 months after the effective date of the final rule, until the applicable final report is submitted to EPA.

(C) Interim progress reports shall be submitted to EPA for the heritable translocation assay at 6-month intervals beginning 6 months after the date of EPA's notification of the test sponsor that testing shall be initiated, until the final report is submitted to EPA.

(7) *Neutrotoxicity*—(i) *Required testing.* (A)(*1*) A schedule-controlled operant behavior test shall be conducted with commercial hexane in accordance with § 798.6500 of this chapter except for the provisions in paragraphs (d)(5)(i), (6) and (7) of § 798.6500.

(*2*) For the purposes of this section, the following provisions also apply:

(*i*) *High dose level.* The highest dose shall produce clear behavioral effects or life-threatening toxicity. In addition, the highest dose should not exceed the lower explosive limit of commercial hexane.

(*ii*) *Duration and frequency of exposure.* Animals shall be dosed once for 4 to 6 hours.

(*iii*) *Route of administration.* Animals shall be exposed to commercial hexane by inhalation.

(B)(*1*) A functional observation battery shall be conducted with commercial hexane in accordance with § 798.6050 of this chapter except for the provisions in paragraphs (d)(4)(i), (5), and (6) of § 798.6050.

(*2*) For the purposes of this section, the following provisions also apply:

(*i*) *High dose level.* The highest dose shall produce clear behavioral effects or life-threatening toxicity. In addition, the highest dose should not exceed the lower explosive limit of commercial hexane.

(*ii*) *Duration and frequency of exposure.* Animals shall be dosed for 6 hours/day, 5 days/week for 90 days.

(*iii*) *Route of exposure.* Animals shall be exposed to commercial hexane by inhalation.

(C)(*1*) A motor activity test shall be conducted with commercial hexane in accordance with § 798.6200 of this chapter except for the provisions in paragraphs (d)(4)(i), (5), and (6) of § 798.6200.

(*2*) For the purposes of this section, the following provisions also apply:

(*i*) *High dose level.* The highest dose shall produce clear effects on motor activity of life-threatening toxicity. In addition, the highest dose should not exceed the lower explosive limit of commercial hexane.

(*ii*) *Duration and frequency of exposure.* Animals shall be dosed for 6 hours/day, 5 days/week for 90 days.

(*iii*) *Route of exposure.* Animals shall be exposed to commercial hexane by inhalation.

(D)(*1*) A neuropathology test shall be conducted with commercial hexane in accordance with § 798.6400 of this chapter except for the provisions in paragraphs (d)(4)(i), (5), and (6) of § 798.6400.

(*2*) For the purposes of this section, the following provisions also apply:

(*i*) *High dose level.* The highest dose shall produce clear behavior effects or life-threatening toxicity. In addition, the highest dose should not exceed the lower explosive limit of commercial hexane.

(*ii*) *Duration and frequency of exposure.* Animals shall be dosed for 6 hours/day, 5 days/week for 90 days.

(*iii*) *Route of exposure.* Animals shall be exposed to commercial hexane by inhalation.

(ii) *Reporting requirements.* (A) The schedule-controlled operant behavior, functional observation battery, motor activity, and neuropathology tests shall be completed and the final reports submitted to EPA within 15 months of the effective date of the final rule.

(B) Interim progress reports for each test shall be submitted to EPA for the schedule-controlled operant behavior, functional observation battery, motor activity, and neuropathology tests at 6-month intervals beginning 6 months after the effective date of the applicable final rule, until the applicable final report is submitted to EPA.

(8) [Reserved]

(d) *Effective date.* (1) The effective date of § 799.2155 will be November 17, 1988.

(2) The guidelines and other test methods cited in this section are referenced as they exist on the effective date of the final rule.

(Information collection requirements have been approved by the Office of Managment and Budget under control number 2070-0033)

[53 FR 3392, Feb. 5, 1988, as amended at 53 FR 38953, Oct. 4, 1988]

### § 799.2175 C9 aromatic hydrocarbon fraction.

(a) *Identification of chemical substance.* The C9 aromatic hydrocarbon fraction obtained from the reforming of crude petroleum shall be tested in accordance with this part.

(b) *Identification of test substance.* A C9 substance consisting of *ortho-*, *meta-* and *para*-ethyltoluene (minimum 22 percent), and 1,2,4-, 1,2,3,- and 1,3,5-trimethylbenzene minimum 15 percent) that is representative of a typical C9 aromatic hydrocarbon fraction obtained from the reforming of crude petroleum (minimum total ET-TMB content 75 percent) and intended for use as a solvent end product shall be prepared and used as the test substance in all tests.

(c) *Persons required to submit study plans, conduct tests and submit data.* All persons who manufacture or process, or intend to manufacture or process, the C9 aromatic hydrocarbon fraction, other than as an impurity, from July 1, 1985, to the end of the reimbursement period shall submit letters of intent to test, exemption applications, and study plans, and shall conduct tests and submit data as specified in this section, Subpart A of this part, and Part 790 of this chapter.

(d) *Health Effects Testing*—(1) *Mutagenic effects—Chromosomal aberrations*—(i) *Required testing.* (A) An in vitro cytogenetics test shall be conducted with the C9 test substance.

(B) An in vivo cytogenetics test shall be conducted for the C9 test substance if the in vitro cytogenetics test conducted pursuant to paragraph (d)(1)(i)(A) of this section produces a negative result.

(C) A dominant lethal assay shall be conducted with the C9 test substance unless the in vitro cytogenetics test conducted pursuant to paragraph (d)(1)(i)(A) of this section and the in vivo cytogenetics test conducted pursuant to paragraph (d)(1)(i)(B) of this section (if required) produce negative results.

(D) A heritable translocation assay shall be conducted with the C9 test substance if the dominant lethal assay conducted pursuant to paragraph (d)(1)(i)(C) of this section produces a positive result.

(ii) *Reporting requirements.* (A) The mutagenic effects testing for chromosomal aberrations as contained in the first tier of testing, which consists of an *in vitro* cytogenetics test and an *in vivo* cytogenetics test shall be completed and the final results submitted to the Agency within 12 months of the effective date of the final Phase II rule.

(B) The mutagenic effects testing for chromosomal aberrations as contained in the second tier of testing, which consists of a dominant lethal assay, shall be completed and the final results submitted to the Agency within 24 months of the effective date of the final Phase II rule.

(C) The mutagenic effects testing for chromosomal aberrations as contained in the third tier of testing, which consists of a heritable translocation assay, shall be completed and the final results submitted to the Agency within 24 months of the date of EPA's notification of the test sponsor by certified letter or FEDERAL REGISTER notice that testing should be initiated.

(D) Progress reports shall be submitted to the Agency for the *in vitro* and *in vivo* cytogenetics assays and the dominant lethal assay at 6-month intervals, the first of which is due within 6 months of the effective date of the final Phase II rule.

(E) Progress reports shall be submitted to the Agency for the heritable translocation assay at 6-month intervals, the first of which is due within 6 months of the date of EPA's notification of the test sponsor that testing should be initiated.

(2) *Mutagenic effects—Gene mutation*—(i) *Required testing.* (A) A *Salmonella typhimurium* mammalian reverse mutation microsomal assay shall be conducted with the C9 test substance, both with and without activation.

(B) A sister chromatid exchange (SCE) assay shall be conducted with the C9 test substance.

(C) A gene mutation in mammalian cells in culture assay shall be conducted with the C9 test substance.

(D) A second gene mutation in mammalian cells in culture assay, using a different cell line from that used in the first assay, shall be conducted with the C9 test substance if the first gene mutation in cells in culture assay, conducted pursuant to paragraph (d)(2)(i)(C) of this section, produces a negative result, unless the *Salmonella* microsomal assay, conducted pursuant to paragraph (d)(2)(i)(A) of this section, and the SCE assay, conducted pursuant to paragraph (d)(2)(i)(B) of this section, produce negative results.

(E) A *Drosophila* sex-linked recessive lethality test shall be conducted with the C9 test substance unless the *Salmonella* microsomal assay conducted pursuant to paragraph (d)(2)(i)(A) of this section and the gene mutation in cells in culture assays conducted pursuant to paragraphs (d)(2)(i)(C) and (D) of this section produce negative results.

(F) A mouse specific locus assay shall be conducted with the C9 test substance if the *Drosophila* sex-linked recessive lethality test, conducted pursuant to paragraph (d)(2)(i)(E) of this section produces a positive result.

(ii) *Reporting requirements.* (A) The mutagenic effects testing for gene mutations as contained in the first tier of testing, which consists of a *Salmonella typhimurium* mammalian reverse mutation microsomal assay, a sister chromatid exchange (SCE) assay, and a gene mutation in mammalian cells in culture assay, shall be completed and the final results submitted to the Agency within 12 months of the effective date of the final Phase II rule.

(B) The mutagenic effects testing for gene mutations as contained in the second tier of testing, which consists of a second gene mutation in mammalian cells in culture assay and a *Drosophila* sex-linked recessive lethal test, shall be completed and the final results submitted to the Agency within 24 months of the effective date of the final Phase II rule.

(C) The mutagenic effects testing for gene mutations as contained in the third tier of testing, consisting of a mouse specific locus assay, shall be completed and the final results submitted to the Agency within 48 months of the date of EPA's notification of the test sponsor by certified letter or FEDERAL REGISTER notice that testing should be initiated.

(D) Progress reports shall be submitted to the Agency for the *Salmonella typhimurium* mammalian reverse mutation microsomal assay, SCE assay, gene mutation in mammalian cells in culture assays, and *Drosophila* sex-linked recessive lethal test at 6-month intervals, the first of which is due within 6 months of the effective date of the final Phase II rule.

(E) Progress reports shall be submitted to the Agency for the mouse specific locus assay at 6-month intervals, the first of which is due within 6 months of the date of EPA's notification of the test sponsor that testing should be initiated.

(3) *Oncogenicity*—(i) *Required testing.* A 2-year inhalation oncogenicity bioassay shall be conducted with the C9 test substance unless it produces negative results in all of the following tests: In vitro cytogenetics test, in vivo cytogenetics test (if required), first gene mutation in cells in culture assay, second gene mutation in cells in culture assay (if required), and *Drosophila* sex-linked recessive lethality test (if required) conducted pursuant to paragraphs (d)(1)(i)(A) and (B) and (d)(2)(i)(C), (D) and (E) of this section.

(ii) *Reporting requirements.* (A) The oncogenicity testing shall be completed and the final results submitted to the Agency within 53 months after the date of EPA's notification of the test sponsor by certified letter or FEDERAL REGISTER notice that testing should be initiated.

(B) Progress reports shall be submitted to the Agency at 6-month intervals, the first of which is due within 6 months after the date of EPA's notification of the test sponsor that testing should be initiated.

(4) *Developmental Toxicity*—(i) *Required testing.* An inhalation developmental toxicity study shall be conducted with the C9 test substance.

(ii) *Reporting requirements.* (A) The developmental toxicity testing shall be completed and the final results submitted to the Agency within 18 months of the effective date of the final Phase II rule.

(B) Progress reports shall be submitted to the Agency at 6-month intervals, the first of which is due within 6 months from the effective date of the final Phase II rule.

(5) *Reproductive Effects*—(i) *Required testing.* An inhalation reproductive effects study shall be conducted with the C9 test substance.

(ii) *Reporting requirements.* (A) The reproductive effects testing shall be completed and the final results submitted to the Agency within 29 months of the effective date of the final Phase II rule.

(B) Progress reports shall be submitted to the Agency at 6-month intervals, the first of which is due within 6 months from the effective date of the final Phase II rule.

(6) *Neurotoxicity*—(i) *Required testing.* A neurotoxicity test battery consisting of a 90-day subchronic inhalation exposure incorporating the following tests shall be conducted with the C9 test substance:

(A) A neuropathology test;

(B) A motor activity test; and

(C) A functional observation battery.

(ii) *Reporting requirements.* (A) The neurotoxicity test battery consisting of a motor activity test and functional observational battery shall be completed and the final results submitted to the Agency within 15 months from the effective date of the final Phase II rule.

(B) The neuropathology test shall be completed and the final results submitted to the Agency within 25 months from the effective date of the final Phase II rule.

(C) Progress reports shall be submitted to the Agency at 6-month intervals, the first of which shall be due within 6 months from the effective date of the final Phase II rule.

(e) *Test standards*—(1) *General.* (i)(A) The required testing specified in paragraphs (d) (1), (2), (3), and (4) of this section shall be conducted in accordance with the study plans for testing the C9 fraction developed by the American Petroleum Institute (API), submitted to EPA on September 30, 1985, modified in a submission dated January 10, 1986, and the additional requirements specified in this paragraph.

(B) The required testing specified in paragraph (d)(5) of this section shall be conducted in accordance with the study plans for testing the C9 fraction developed by the American Petroleum Institute (API), submitted to EPA on September 30, 1985, and modified in submissions dated January 10, 1986, and September 13, 1988.

(ii) The required testing specified in paragraph (d)(6) of this section shall be conducted in accordance with the study plan for testing the $C_9$ fraction developed by API, and submitted to the Agency on November 4, 1986.

(iii) Copies of the API study plans are located in the public record for this rule (Docket No. OPTS-42034) and are available for inspection in EPA's OPTS Reading Rm., NE-G004, 401 M St., SW., Washington, DC 20460, from 8 a.m. to 4 p.m., Monday through Friday, except legal holidays.

(2) *Mutagenic effects.* (i) For each study specified in paragraphs (d)(1)(i)(A) and (2)(i)(A), (B), (C), and (D) of this section, the study shall be repeated over a narrow range of concentrations if a single, statistically significant positive effect for at least one of the test points is produced where no statistically significant dose-related increase in the number of mutagenic events was found.

(ii) For each study specified in paragraph (d) of this section, in addition to the criteria for determining a positive result given in the study plans specified in paragraph (e)(1) of this section, the detection of a reproducible and statistically significant response for at least one of the test substance concentrations shall be interpreted as a positive result. In the absence of a repeat assay, a statistically significant response for at least one of the test substance concentrations shall be interpreted as a positive response.

(iii) For the mouse heritable translocation assay specified in paragraph (d)(1)(i)(D) of this section, the following are required.

(A) If the laboratory's historical control data base is inadequate, concurrent positive and negative controls shall be conducted which conform to the requirements specified in § 798.5200(d)(4)(i) of this chapter.

(B) Control data shall be presented, whether they are historical or concurrent, in the final report of the study and shall be identified as either the one or the other.

(3) *Oncogenicity*—(i) *Dose levels and dose selection.* The lowest dose shall not be lower than 10 percent of the high dose.

(ii) *Duration.* Each study shall last the majority of the normal lifespan of the strain of animals to be used. This time period shall not be less than 24 months for rats and 18 months for mice, and ordinarily not longer than 30 months for rats and 24 months for mice.

(iii) *Histopathology.* Target organs (including but not limited to lungs and respiratory tract) in all animals shall be subject to a histopathological examination.

(iv) *Individual animal data.* (A) Food and water consumption data shall be reported, when measured.

(B) Ophthalmological data shall be recorded when the examination is performed.

(4) *Developmental toxicity.* (i) Testing in one mammalian species other than the rat is required.

(ii) Dams shall be killed before parturition.

(5) *Test substance*—(i) *Identity and source.* The remaining components, which may be as high as 25 percent of the test mixture, shall be characterized.

(ii) *Stability under test and storage conditions.* The atmosphere being inhaled by the animals shall be characterized with regard to concentration and identification of the components inhaled.

(f) *Effective date.* The effective date of the final Phase II rule for the $C_9$ aromatic hydrocarbon fraction is March 9, 1987.

(Information collection requirements approved by the Office of Management and Budget under control number 2070-0033)

[50 FR 20676, May 17, 1985, as amended at 52 FR 2527, Jan. 23, 1987; 54 FR 27357, June 29, 1989]

### § 799.2200 Hydroquinone.

(a) *Identification of test substance.* (1) Hydroquinone (CAS No. 123-31-9) shall be tested in accordance with this section.

(2) Hydroquinone of at least 99 percent purity shall be used as the test substance.

(b) *Persons required to submit study plans, conduct tests and submit data.* (1) All persons who manufacture or process hydroquinone, other than as an impurity, from January 13, 1986 to the end of the reimbursement period shall submit letters of intent to test, exemption applications, and shall conduct tests and submit data as specified in this section, Subpart A of this part and Part 790 of this chapter for two-phase rulemaking.

(2) Persons subject to this section are not subject to the requirements of § 790.50(a)(2), (5), (6), and (b), and § 790.87(a)(1)(ii) of this chapter.

(3) Persons who notify EPA of their intent of conduct tests in compliance with the requirements of this section must submit plans for those tests no later than 30 days before the initiation of each of those tests.

(4) In addition to the requirements of § 790.87(a)(2) and (3) of this chapter, EPA will conditionally approve exemption applications for this rule if EPA has received a letter of intent to conduct the testing from which exemption is sought and EPA has adopted test standards and schedules in a final Phase II test rule.

(c) *Health effects testing*—(1) *Toxicokinetic studies*—(i) *Required testing.* Skin and oral dosing studies, which will provide data regarding both rate and extent of absorption, shall be conducted with hydroquinone.

(ii) *Test standard.* (A) The toxicokinetic testing shall be conducted in accordance with § 795.235 of this chapter except for the provisions in paragraph (c)(1)(iii)(C) of § 795.235.

(B) For the purpose of this section, the following provisions also apply:

(*1*) During the acclimatization period, rats shall be housed in polycarbonate cages on hardboard chip bedding, or suspended steel cages with no bedding material.

(*2*) [Reserved]

(iii) *Reporting requirements.* (A) The toxicokinetic tests shall be completed and the final results submitted to the Agency within 1 year of the effective date of the Phase II final test rule.

(B) A progress report shall be provided 6 months from the effective date of the final Phase II rule.

(2) *Developmental Toxicity*—(i) *Required testing.* Developmental toxicity studies in both a rodent and nonrodent species shall be conducted with hydroquinone. These tests must be conducted using the oral route of exposure.

(ii) *Test standards.* The developmental toxicity testing shall be conducted in accordance with § 798.4900, as revised July 1, 1987.

(iii) *Reporting requirements.* (A) The Developmental toxicity tests shall be completed and the final results submitted to the Agency within 18 months of the effective date of the final Phase II rule.

(B) Interim progress reports shall be provided at 6-month intervals beginning 6 months from the effective date of the final Phase II rule.

(3) *Reproductive Effects*—(i) *Required testing.* A two-generation reproductive effects study in a rodent species shall be conducted with hydroquinone. This test must be conducted using the oral route of exposure.

(ii) *Test standards.* The reproductive effects testing shall be conducted in accordance with § 798.4700, as revised July 1, 1987.

(iii) *Reporting requirements.* (A) The two-generation reproductive effects toxicity test shall be completed and final results submitted to the Agency within 29 months of the effective date of the final Phase II rule.

(B) Interim progress reports shall be provided at 6-month intervals beginning 6 months from the effective date of the final Phase II rule.

(4) *Neurotoxicity*—(i) *Required testing.* The following neurotoxicity testing shall be conducted for hydroquinone using oral exposure of a rodent species:

(A) A functional observational battery.

(B) A neuropathology test.

(ii) *Test standards.* (A) The neurotoxicity testing of hydroquinone, consisting of a functional observational battery and neuropathology, shall be conducted in accordance with §§ 798.6050 and 798.6400, respectively, of this chapter, except for the provisions of paragraphs (d)(8) (ii) (C) and (D), (iv) (A), and (E)(2) of § 798.6400. The functional-observational battery and the neuropathology assessment may be conducted sequentially on the same group of rats. Neuropathological assessment should begin with the highest dose level and work downward until a no-observable-adverse-effects dose is reached.

(B) For the purpose of § 798.6400, the following provisions also apply:

(*1*) *Removal of brain and cord.* After perfusion, the bony structure (cranium and vertebral column) should be exposed. Animals should then be stored in fixative-filled bags at 4 °C for 8–12 hours. The cranium and vertebral column shall be removed carefully by trained technicians without physical damage of the brain and cord. Detailed dissection procedures may be found in the text by Palay and Chan-Palay (1974) under paragraph (f)(4) of this section. After removal, simple measurement of the weight of the whole brain (cerebrum, cerebellum, pons-medulla) should be made. Any abnormal coloration or discoloration of the brain and cord should also be noted and recorded.

(*2*) *Sampling.* Unless a given test rule specifies otherwise, cross-sections of the following areas shall be examined: The forebrain, the center of the cerebrum, the midbrain, the cerebellum and pons, and the medulla oblongata; the spinal cord at cervical and lumbar swelling (C3-C6 and L1-L4); dorsal root ganglia (C3-C6 and L1-L4), dorsal and ventral root fibers (C3-C6 and L1-L4), sciatic nerve (mid-thigh) and tibial nerve (at knee). The aforementioned areas will be examined with special stains (a combined Luxol Fast Blue Stain-Bodian Silver Protargol impregnation).

(*3*) *Histopathology examination.* Tissue specimens stored in 10 percent buffered formalin may be used for this purpose. All tissues must be immersion-fixed in fixative for at least 48 hours prior to further tissue processing. Alternative fixation procedures may be employed. Tissues for plastic embedment may be fixed for an additional period of at least 2 hours in glutaraldehyde. Tissues from perfused animals not destined for plastic embedment and all tissues from unperfused animals may be fixed in 10 percent neutral buffered formalin.

(*4*) *Special stains.* Regardless of the results of the general staining, selected sites and cellular components shall be further evaluated by the use of certain special stains (a combined Luxol Fast Blue Stain-Bodian Silver Protargol impregnation) and plastic embedded 1 micron sections. These stains and sections shall be used to detect chemical-induced damage to neuronal body, axon, myelin sheath and neurofibrils. A section of normal tissue shall be included in each staining to assure that adequate staining has occurred. Any changes shall be noted and representative photographs shall be taken. If a lesion(s) is observed, the special techniques shall be repeated in the next lower treatment group until no further lesion is detectable.

(iii) *Reporting requirements.* (A) The neurotoxicity tests shall be completed and final results submitted to EPA within 16 months of the effective date of the final Phase II rule.

(B) Interim progress reports shall be provided 6 months from the effective date of the final Phase II rule.

(d) *Effective date.* The effective date of the final Phase II rule for hydroquinone is July 13, 1987.

(Information collection requirements have been approved by the Office of Management and Budget under control number 2070-0033)

[50 FR 53156, Dec. 30, 1985, as amended at 52 FR 19870, May 28, 1987; 54 FR 27358, June 29, 1989]

### § 799.2475 2-Mercaptobenzothiazole.

(a) *Identification of test substance.* (1) 2-Mercaptobenzothiazole (MBT, CAS No. 149-30-4) shall be tested in accordance with this section.

(2) MBT of at least 98 percent purity shall be used as the test substance.

(b) *Persons required to submit study plans, conduct tests, and submit data.* All persons who manufacture (including byproduct manufacture, and import of MBT and MBT-containing articles) or process or intend to manufacture or process MBT, other than as an impurity, after October 21, 1988, to the end of the reimbursement period shall submit letters of intent to conduct testing, submit study plans, conduct tests, and submit data, or submit exemption applications as specified in this section, Subpart A of this Part, and Parts 790 and 792 of this chapter for single-phase rulemaking.

(c) *Chemical fate*—(1) *Aerobic aquatic biodegradation*—(i) *Required testing.* Aerobic aquatic biodegradation testing shall be conducted with MBT in accordance with § 796.3100 of this chapter.

(ii) *Reporting requirements.* (A) The aerobic aquatic biodegradation test shall be completed and the final report submitted to EPA within 12 months of the effective date of the final rule.

(B) An interim progress report shall be submitted to EPA 6 months after the effective date of the final rule.

(2) *Indirect photolysis-screening level test*—(i) *Required testing.* Indirect photolysis testing shall be conducted with MBT in accordance with § 795.70 of this chapter.

(ii) *Reporting requirements.* (A) The indirect photolysis test shall be completed and the final report submitted to EPA within 12 months of the effective date of the final rule.

(B) An interim progress report shall be submitted to EPA 6 months after the effective date of the final rule.

(3) *Chemical mobility*—(i) *Required testing.* Chemical mobility testing shall be conducted with MBT in ac-

cordance with § 796.2750 of this chapter.

(ii) *Reporting requirements.* (A) The chemical mobility test shall be completed and the final report submitted to EPA within 12 months of the effective date of the final rule.

(B) An interim progress report shall be submitted to EPA 6 months after the effective date of this final rule

(d) *Environmental effects*—(1) *Fish chronic toxicity*—(i) *Required testing.* (A) Chronic toxicity testing of MBT shall be conducted using rainbow trout (*Salmo gairdneri*) according to § 797.1600 of this chapter except for the provisions in paragraph (c)(6)(iv)(A) of § 797.1600.

(B) For the purpose of this section, the following provisions also apply:

(*1*) *Test substance measurement.* Prior to addition of the test substance to the dilution water, it is recommended that the test substance stock solution be analyzed to verify the concentration. After addition of the test substance, the concentration of test substance shall be measured in the test substance delivery chamber prior to beginning, and during, the test. The concentration of test substance should also be measured at the beginning of the test in each test concentration (including both replicates) and control(s), and at least once a week thereafter. Equal aliquots of test solution may be removed from each replicate chamber and pooled for analysis. If a malfunction in the delivery system is discovered, water samples shall be taken from the affected test chambers immediately and analyzed.

(*2*) *pH.* It is recommended that a pH of 7 be maintained in the test chambers.

(*3*) *Reporting.* An analysis of the stability of the stock solution for the duration of the test shall be reported.

(ii) *Reporting requirements.* (A) The fish chronic toxicity test shall be completed and the final report submitted to EPA within 12 months of the effective date of the final rule.

(B) An interim progress report shall be submitted to EPA 6 months after the effective date of the final rule.

(2) *Daphnid chronic toxicity*—(i) *Required testing.* (A) Daphnid chronic toxicity testing shall be conducted with MBT using *Daphnia magna* according to § 797.1330 of this chapter.

(B) For the purposes of this section, the following provisions also apply:

(*1*) *Test substance measurement.* Test substance concentration shall be measured in the test substance delivery chamber prior to beginning, and during, the test.

(*2*) *pH.* It is recommended that a pH of 7 be maintained in the test chambers.

(*3*) *Reporting.* An analysis of the stability of the stock solution for the duration of the test shall be reported.

(ii) *Reporting requirements.* (A) The daphnid chronic toxicity test shall be completed and the final report submitted to EPA within 12 months of the effective date of the final rule.

(B) An interim progress report shall be submitted to EPA 6 months after the effective date of the final rule.

(e) *Health effects*—(1) *Developmental toxicity testing*—(i) *Required testing.* Developmental toxicity testing shall be conducted in two mammalian species with MBT in accordance with § 798.4900 of this chapter, using the oral route of administration.

(ii) *Reporting requirements.* (A) The developmental toxicity test shall be completed and the final report submitted to EPA within 12 months of the effective date of the final rule.

(B) An interim progress report shall be submitted to EPA 6 months after the effective date of the final rule.

(2) *Reproductive toxicity*—(i) *Required testing.* Reproductive toxicity testing shall be conducted with MBT in accordance with § 798.4700 of this chapter, using the oral route of administration.

(ii) *Reporting requirements.* (A) The reproductive test shall be completed and the final report submitted to EPA within 29 months of the effective date of the final rule.

(B) Progress reports shall be submitted to EPA at 6-month intervals beginning 6 months after the effective date of the final rule until submission of the final report.

(3) *Neurotoxicity*—(i) *Required testing.* (A)(*1*) An acute and subchronic functional observation battery shall be conducted with MBT in accordance with § 798.6050 of this chapter except

for the provisions in paragraphs (d)(5) and (6) of § 798.6050.

(*2*) For the purpose of this section, the following provisions also apply:

(*i*) *Duration and frequency of exposure.* For acute study, animals shall be administered MBT over a period not to exceed 24 hours. For subchronic study, animals shall be dosed daily for at least 90 days.

(*ii*) *Route of exposure.* Animals shall be exposed to MBT orally.

(B)(*1*) An acute and subchronic motor activity test shall be conducted with MBT in accordance with § 798.6200 of this chapter except for the provisions in paragraphs (d)(5) and (6) of § 798.6200.

(*2*) For the purpose of this section the following provisions also apply:

(*i*) *Duration and frequency of exposure.* For acute study, animals shall be administered over a period not to exceed 24 hours. For subchronic study, animals shall be dosed daily for at least 90 days.

(*ii*) *Route of exposure.* Animals shall be exposed to MBT orally.

(C)(*1*) A subchronic neuropathology test shall be conducted with MBT in accordance with § 798.6400 of this chapter except for the provisions in paragraphs (d)(5) and (6) of § 798.6400.

(*2*) For the purpose of this section, the following provisions also apply:

(*i*) *Duration and frequency of exposure.* Animals shall be dosed daily for at least 90 days.

(*ii*) *Route of exposure.* Animals shall be exposed to MBT orally.

(ii) *Reporting requirements.* (A) The functional observation battery, motor activity, and neuropathology tests shall be completed and the final reports for each test submitted to EPA within 12 months of the effective date of the final rule.

(B) A progress report shall be submitted to EPA for the functional observation battery, motor activity, and neuropathology tests, respectively, 6 months after the effective date of the final rule.

(4) *Mutagenic effects—Chromosomal aberrations*—(i) *Required testing.* (A) A dominant lethal assay shall be conducted with MBT in accordance with § 798.5450 of this chapter, using the oral route of administration.

(B) A heritable translocation assay shall be conducted with MBT in accordance with the test guideline specified in § 798.5460 of this chapter if MBT produces a positive result in the dominant lethal assay conducted pursuant to paragraph (e)(4)(i)(A) of this section and if, after a public program review, EPA issues a FEDERAL REGISTER notice or sends a certified letter to the test sponsor specifying that the testing shall be initiated.

(ii) *Reporting requirements.* (A) Mutagenic effects—Chromosomal aberration testing of MBT shall be completed and the final report submitted to EPA as follows: Dominant lethal assay, within 12 months after the effective date of this rule; heritable translocation assay, within 24 months after notification under paragraph (e)(4)(i)(B) of this section that the testing shall be initiated.

(B) For the dominant lethal assay, an interim progress report shall be submitted to EPA 6 months after the effective date of the final rule; for the heritable translocation assay, progress reports shall be submitted to EPA at 6-month intervals beginning 6 months after the date of EPA's notification of the test sponsor that testing shall be initiated until submission of the final report.

(f) *Effective date.* (1) The effective date of this final rule is October 21, 1988.

(2) The guidelines and other test methods cited in this section are referenced here as they exist on October 21, 1988.

(Information collection requirements have been approved by the Office of Management and Budget under Control Number 2070-0033)

[53 FR 34530, Sept. 7, 1988; 53 FR 37393, Sept. 26, 1988]

### § 799.2500 Mesityl oxide (MO).

(a) *Identification of test substance.* (1) Mesityl oxide (CAS No. 141-79-7) shall be tested in accordance with this section.

(2) Mesityl oxide of at least 97 percent purity shall be used as the test substance.

(b) *Persons required to submit study plans, conduct tests, and submit data.* (1) All persons who manufacture or process or intend to manufacture or process MO from the effective date of this rule, February 3, 1986, to the end of the reimbursement period shall submit letters of intent to conduct testing or exemption applications, study plans, and/or shall conduct tests, and submit data as specified in this section, Subpart A of this part, and Part 790 of this chapter.

(2) Persons subject to this section are not subject to the requirements of § 790.50(a)(2), (5), and (6) and (b), and § 790.87(a)(1)(ii) of this chapter.

(3) Persons who notify EPA of their intent to conduct tests in compliance with the requirements of this section must submit plans for these tests no later than 30 days before the initiation of each of those tests.

(4) In addition to the requirements of § 790.87(a)(2) and (3) of this chapter, EPA will conditionally approve exemption applications for this rule if EPA has received a letter of intent to conduct the testing from which exemption is sought and EPA has adopted test standards and schedules in a final Phase II test rule.

(c) *Health effects testing*—(1) *Subchronic inhalation toxicity*—(i) *Required testing.* A 90-day subchronic inhalation toxicity test shall be conducted with MO.

(ii) *Test standard.* Inhalation subchronic toxicity testing shall be conducted with MO in accordance with § 798.2450 of this chapter, except for the provisions of § 798.2450 (d)(1)(i) and (d)(11)(i)(A).

(iii) For the purposes of this section the following provisions also apply:

(A) *Animal Selection—species and strain.* The rat shall be used. Commonly used laboratory strains should be employed. The tester should provide justification/reasoning for its selection.

(B) *Clinical examinations.* Certain hematological determinations shall be carried out at least three times during the test period: just prior to initiation of dosing (base line data), after approximately 30 days on test, and just prior to terminal sacrifice at the end of the test period. Hematology determinations which shall be appropriate to all studies include the following: Hematocrit, hemoglobin concentration, erythrocyte count, total and differential leucocyte count, and a measure of clotting potential such as clotting time, prothrombin time, thromboplastin time, or platelet count.

(iv) *Reporting requirements.* (A) The subchronic testing shall be completed and the final results submitted to the Agency within 15 months of the effective date of the final Phase II test rule.

(B) Progress reports shall be provided every 6 months beginning 6 months after the effective date of the final Phase II test rule.

(2) *Mutagenic effects—chromosomal aberrations*—(i) *Required testing.* (A) An *in vitro* cytogenetic test shall be conducted with MO.

(B) An *in vivo* cytogenetic test shall be conducted for MO if the *in vitro* cytogenetic test conducted pursuant to paragraph (c)(2)(i)(A) of this section produces a negative result.

(C) A dominant lethal assay shall be conducted for MO if it produces a positive result in the *in vivo* or *in vitro* cytogenetics test conducted pursuant to paragraphs (c)(2)(i)(A) and (B) of this section.

(D) A heritable translocation assay shall be conducted for MO if it produces a positive result in the dominant lethal assay conducted pursuant to paragraph (c)(2)(i)(C) of this section.

(ii) *Test standard.* (A) (*1*) The *in vitro* mammalian cytogenetics test shall be conducted with MO in accordance with § 798.5375 of this chapte except for the provisions in § 798.537 (d)(3)(i) and (d)(6)(ii).

(*2*) For the purposes of this section the following provisions also apply:

(*i*) *Type of cells used in the assa* MO shall be tested in established cel lines. The cell line or strain used shal be checked for *Mycoplasma* contamination and for karyotype stability.

(*ii*) *Exposure concentrations.* A least 3 concentrations of the test substance over a range adequate to defin the response shall be tested. The highest test concentration tested with an without metabolic activation shall be milligrams per milliliter or that do which shows evidence of cytotoxicity or reduced mitotic activity.

(B)(*1*) The *in vivo* mammalian bone marrow cytogenetics test: Chromosomal analysis shall be conducted with MO in accordance with § 798.5385 of this chapter except for the provisions in § 798.5385(d)(5) (ii) and (iii).

(*2*) For the purposes of this section the following provisions also apply:

(*i*) *Dose levels.* Three dose levels shall be used. The highest dose tested shall be the maximum tolerated dose or that producing some indication of cytotoxicity (e.g., partial inhibition of mitosis), or shall be the highest dose attainable.

(*ii*) *Route of administration.* The animals shall be exposed by inhalation for 6 hours/day for 5 consecutive days.

(C)(*1*) The rodent dominant lethal assay shall be conducted with MO in accordance with § 798.5450 of this chapter except for the provisions in § 798.5450(d)(5) (ii) and (iii).

(*2*) For the purposes of this section the following provisions also apply:

(*i*) *Dose levels.* Three dose levels shall be used. The highest dose shall produce signs of toxicity (e.g., slightly reduced fertility or body weight) or shall be the highest attainable.

(*ii*) *Route of administration.* Exposure shall be by inhalation for 5 days for 6 hours/day.

(D)(*1*) The rodent heritable translocation test shall be conducted with MO in accordance with § 798.5460 of this chapter except for the provisions in § 798.5460(d)(5) (ii) and (iii).

(*2*) For the purposes of this section the following provisions also apply:

(*i*) *Dose levels.* At least two dose levels shall be used. The highest dose shall result in toxic effects (which shall not produce an incidence of fatalities which would prevent a meaningful evaluation), or shall be the highest dose attainable.

(*ii*) *Route of administration.* Animals shall be exposed by inhalation.

(iii) *Reporting requirements.* (A) The chromosomal aberration tests shall be completed and the final results submitted to the Agency as follows:

(*1*) The *in vitro* and *in vivo* (conditional) tests within 15 months of the effective date of the final Phase II test rule.

(*2*) The dominant lethal assay (conditional) within 24 months of the effective date of the final Phase II test rule.

(*3*) The heritable translocation test (conditional) within 24 months of the date of EPA's notification of the test sponsor by certified letter or FEDERAL REGISTER notice that testing shall be initiated.

(B) Progress reports shall be submitted to the Agency for the *in vitro* and *in vivo* cytogenetics assays and the dominant lethal assay at 6-month intervals, the first of which is due within 6 months of the effective date of the final Phase II rule.

(C) Progress reports shall be submitted to the Agency for the heritable translocation assay at 6-month intervals, the first of which is due within 6 months of the date of EPA's notification of the test sponsor that testing shall be initiated.

(3) *Mutagenic effects—gene mutations*—(i) *Required testing.* (A) A *Salmonella typhimurium* mammalian microsomal reverse mutation assay (Ames assay) shall be conducted with MO.

(B) A sex-linked recessive lethal test in *Drosophila melanogaster* shall be conducted for MO if it produces a positive result in the Ames assay conducted pursuant to paragraph (c)(3)(i)(A) of this section.

(C) A gene mutation in somatic cells assay shall be conducted with MO if it produces a negative result in the Ames assay conducted pursuant to paragraph (c)(3)(i)(A) of this section.

(D) A sex-linked recessive lethal test in *Drosophila melanogaster* shall be conducted for MO if it produces a positive result in the gene mutation in somatic cells assay conducted pursuant to paragraph (c)(3)(i)(C) of this section.

(E) A mouse specific-locus test shall be conducted for MO if it produces a positive result in the sex-linked recessive-lethal test in *Drosophila melanogaster* conducted pursuant to paragraph (c)(3)(i)(B) or (D) of this section.

(ii) *Test standards*—(A)(*1*) The *Salmonella typhimurium* mammalian microsomal reverse mutation assay (Ames assay) shall be conducted with

MO in accordance with § 798.5265 of this chapter except for the provisions in § 798.5265 (d)(5)(ii), (d)(6)(ii) (A) and (B), and (e)(1).

(*2*) For the purposes of this section the following provisions also apply:

(*i*) *Strain specific positive controls.* Strain specific positive controls shall be included in the assay. The following controls are examples of those which may be used in the assay without metabolic activation: Strain TA 1535, sodium azide; strain TA 100, nitrofurantoin; strains TA 98 and TA 1537, 4-nitro-*o*-phenylenediamine.

(*ii*) *Exposure concentrations.* The test should initially be performed over a broad range of concentrations. Among the criteria to be taken into consideration for determining the upper limits of test chemical concentration are cytotoxicity and solubility. Cytotoxicity of the test chemical may be altered in the presence of metabolic activation systems. Toxicity may be evidenced by a reduction in the number of spontaneous revertants, a clearing of the background lawn or by the degree of survival of treated cultures. Relatively insoluble compounds should be tested up to the limits of solubility. For freely soluble nontoxic chemicals, the upper test chemical concentration should be determined on a case by case basis. MO shall be tested up to 5 milligrams per plate or to the limits of solubility or toxicity. A suspected positive response not showing a clear dose-related response shall be confirmed by testing over a narrow range of concentrations.

(*iii*) *Test performance—Direct plate incorporation method.* The direct plate incorporation method shall be used for this test. For this test without metabolic activation, test chemical and 0.1 milliliter of a fresh bacterial culture should be added to 2.0 milliliter of overlay agar.

(B)(*1*) The detection of gene mutations in somatic cells in culture shall be conducted with MO in accordance with § 798.5300 of this chapter except for the provisions in § 798.5300 (d)(3)(i), (4), and (6)(i) and (e)(1).

(*2*) For the purposes of this section the following provisions also apply:

(*i*) *Types of cells used in the assay.* MO shall be tested at the HGPRT locus in the Chinese hamster ovary cell culture test or in LK5178K mouse lymphoma cells.

(*ii*) *Metabolic activation.* Cells shall be exposed to MO both in the presence and absence of a metabolic activation system derived from the post-mitochondrial fraction (S-9) of livers from rats pretreated with Aroclor 1254.

(*iii*) *Vehicle.* MO may be prepared in culture media or dissolved or suspended in appropriate vehicles prior to treatment of the cells. The final concentration of the vehicle shall not interfere with cell viability or growth rate.

(*iv*) *Test performance.* Cells shall be exposed to MO both with and without exogenous activation. Exposure shall be for 4 hours unless a different exposure time is justified by the investigator.

(C)(*1*) The sex-linked recessive lethal test in *Drosophila melanogaster* shall be conducted with MO in accordance with § 798.5275 of this chapter except for the provisions in paragraph (d)(5)(iii).

(*2*) For the purposes of this section the following provisions also apply: *Route of administration.* Exposure shall be by exposure to MO vapors.

(D)(*1*) The mouse visible specific locus test shall be conducted with MO in accordance with § 798.5200 of this chapter except for the provisions in § 798.5200(d) (5) (ii) and (iii).

(*2*) For the purposes of this section the following provisions also apply:

(*i*) *Dose levels.* A minimum of 2 dose levels shall be tested. Exposure shall be for 6 hours a day. Duration of exposure shall be dependent upon accumulated total dose desired for each group.

(*ii*) *Route of administration.* Animals shall be exposed to MO by inhalation.

(iii) *Reporting requirements*—(A) The gene mutation tests shall be completed and final results submitted to the Agency as follows:

(*1*) The *Salmonella typhimurium* mammalian microsomal reverse mutation assay and the gene mutation in somatic cells assay (conditional) within 12 months of the effective date of the final Phase II test rule.

(*2*) The sex-linked recessive-lethal test in *Drosophila melanogaster* (conditional) within 25 months of the effective date of the final Phase II test rule.

(*3*) The mouse specific-locus test (conditional) within 48 months of the date of EPA's notification of the test sponsor by certified letter or FEDERAL REGISTER notice that testing shall be initiated.

(B) Progress reports shall be submitted to the Agency for the *Salmonella typhimurium* mammalian reverse mutation microsomal assay, gene mutation in mammalian cells in culture assays, and *Drosophila* sex-linked recessive lethal test at 6-month intervals, the first of which is due within 6 months of the effective date of the final Phase II rule.

(C) Progress reports shall be submitted to the Agency for the mouse specific locus assay at 6-month intervals, the first of which is due within 6 months of the date of EPA's notification of the test sponsor that testing shall be initiated.

(4) *Oncogenicity*—(i) *Required testing.* An oncogenicity bioassay shall be conducted by inhalation for MO if MO gives positive results in any one or more of the following tests:

(A) *In vitro* cytogenetics test, conducted pursuant to paragraph (c)(2)(i)(A) of this section.

(B) *In vivo* cytogenetics test, conducted pursuant to paragraph (c)(2)(i)(B) of this section.

(C) Gene mutation in somatic cells assay, conducted pursuant to paragraph (c)(3)(i)(C) of this section.

(D) *Drosophila melanogaster* sex-linked recessive-lethal test, conducted pursuant to paragraph (c)(3)(i)(B) or (D) of this section.

(ii) *Test standard.* (A) (*1*) An oncogenicity bioassay shall be conducted by inhalation with MO in accordance with § 798.3300 of this chapter except for the provisions in § 798.3300(b)(1)(i), (4), and (6).

(*2*) For the purposes of this section the following provisions also apply:

(*i*) *Species and strain.* MO shall be tested in both rats and mice. Commonly used laboratory strains should be employed. The tester should provide justification/reasoning for their selection.

(*ii*) *Exposure conditions.* Animals shall be exposed to MO for at least 6 hours per day on a 5-day per week basis over a period of at least 24 months for rats and 18 months for mice.

(*iii*) *Administration of the test substance.* Animals shall be exposed to MO by the inhalation route.

(B) [Reserved]

(iii) *Reporting requirements.* (A) The oncogenicity tests shall be completed and final results submitted to the Agency 53 months after the date of EPA's notification of the test sponsor by certified letter or FEDERAL REGISTER notice that testing shall be initiated.

(B) Progress reports shall be submitted to the Agency at 6-month intervals, the first of which is due within 6 months after the date of EPA's notification of the test sponsor that testing shall be initiated.

(d) *Effective date.* The effective date of this final Phase II rule for mesityl oxide is July 6, 1987.

(Information collection requirements approved by the Office of Management and Budget under control number 2070–0033)

[50 FR 51866, Dec. 20, 1985; 51 FR 1793, Jan. 15, 1986, as amended at 52 FR 19093, May 20, 1987]

## § 799.3175 Oleylamine.

(a) *Identification of test substance.* (1) 9-Octadecenylamine (hereafter ODA) (CAS Number 112-90-3) shall be tested in accordance with this section.

(2) The ODA test substance shall be at least 90 percent ODA. The vehicle shall be one such as mineral oil for which there are adequate historical toxicological data and which will not interfere in the test results.

(b) *Persons required to submit study plans, conduct tests, and submit data.* (1) All persons who manufacture or process ODA (other than as an impurity) from October 7, 1987 to the end of the reimbursement period shall submit letters of intent to conduct testing or exemption applications, study plans, and/or shall conduct tests in accordance with Part 792 of this chapter, and submit data as specified in this section, Subpart A and Part 790 of this chapter.

(2) Persons subject to this section are not subject to the requirements § 790.50(a)(2), (5), and (6) and (b) and § 790.87(a)(1)(ii) of this chapter.

(3) Persons who notify EPA of their intent to conduct tests in compliance with the requirements of this section must submit plans for those tests no later than 45 days before the initiation of each of those tests.

(4) In addition to the requirements of § 790.87 (a)(2) and (3) of this chapter, EPA will conditionally approve exemption applications for this rule if EPA has received a letter of intent to conduct the testing from which exemption is sought and EPA has adopted test standards and schedules in a final Phase II test rule.

(c) *Health effects testing*—(1) *Developmental toxicity*—(*i*) *Required testing.* An oral developmental toxicity study shall be conducted with ODA in two mammalian species, rat and rabbit.

(ii) *Test standard.* (A) The developmental toxicity study shall be conducted with ODA in accordance with § 798.4900 of this chapter except the provisions of paragraphs (e) (1)(i) and (5) of § 798.4900.

(B) For purposes of this section, the following provisions also apply:

(*1*) *Species and strain.* The rat and rabbit shall be the test species. The strain shall not have low fecundity and shall preferably be characterized for its sensitivity to developmental toxins.

(*2*) *Administration of the test substance.* The route of administration shall be oral by gavage. The test substance shall be administered at approximately the same time each day.

(iii) *Reporting requirements.* (A) The developmental toxicity testing shall be completed and the final report submitted to EPA within 12 months of the date specified in paragraph (d)(1) of this section.

(B) An interim progress report shall be provided to EPA 6 months after the date specified in paragraph (d)(1) of this section.

(2) *Mutagenic effects—chromosomal aberrations*—(i) *Required testing.* (A) An oral *in vivo* mammalian bone marrow cytogenetics test: Chromosomal analysis shall be conducted for ODA.

(B) An oral rodent dominant lethal assay shall be conducted for ODA if it produces a positive result in the *in vivo* mammalian bone marrow cytogenetics test conducted pursuant to paragraph (c)(2)(i)(A) of this section.

(C) An oral rodent heritable translocation assay shall be conducted for ODA if it produces a positive result in the rodent dominant lethal assay conducted pursuant to paragraph (c)(2)(i)(B) of this section and if so required in a FEDERAL REGISTER notice or certified letter sent to test sponsors.

(ii) *Test standard.* (A)(*1*) The *in vivo* mammalian bone marrow cytogenetics test: Chromosomal analysis shall be conducted with ODA in accordance with § 798.5385 of this chapter except the provisions of paragraphs (d) (3)(i) and (5)(iii) of § 798.5385.

(*2*) For purposes of this section, the following provisions also apply.

(*i*) *Species and strain.* Mice shall be used.

(*ii*) *Route of administration.* The route of exposure shall be oral by gavage.

(B)(*1*) The rodent dominant lethal assay shall be conducted with ODA in accordance with § 798.5450 of this chapter except the provisions of paragraphs (d) (3)(i) and (5)(iii) of § 798.5450.

(*2*) For purposes of this section, the following provisions also apply:

(*i*) *Species.* Mice shall be used as the test species. Strains with low background dominant lethality, high pregnancy frequency, and high implant numbers are recommended.

(*ii*) *Route of administration.* The route of administration shall be oral by gavage.

(C)(*1*) The rodent heritable translocation assay shall be conducted with ODA is accordance with § 798.5460 of this chapter, except for the provisions of paragraphs (d) (3)(i) and (5)(iii) of § 798.5460.

(*2*) For purposes of this section, the following provisions also apply.

(*i*) *Species.* Mice shall be used as the test species.

(*ii*) *Route of administration.* The route of administration shall be oral by gavage.

(iii) *Reporting requirements.* (A) The chromosomal aberration tests shall be completed and the final reports submitted to EPA as follows:

(*1*) The *in vivo* mammalian bone marrow cytogenetics test shall be completed within 14 months of the date specified in paragraph (d)(1) of this section.

(*2*) The rodent dominant lethal assay (if required) shall be completed within 26 months of the date specified in paragraph (d)(1) of this section.

(*3*) The rodent heritable translocation assay shall be completed (if required) within 25 months of EPA's notification of the test sponsor by certified letter or FEDERAL REGISTER notice under paragraph (c)(2)(i)(C) of this section that testing should be initiated.

(B) Interim progress reports shall be provided to EPA at 6-month intervals for each test beginning 6 months after the date specified in paragraph (d)(1) of this section or notification that testing should be initiated under paragraph (c)(2)(i)(C) of this section, until submission of the final report.

(3) *Mutagenic effects—gene mutations*—(i) *Required testing.* (A) A detection of gene mutation in somatic cells in culture assay shall be conducted with ODA.

(B) An oral sex linked recessive lethal test in *Drosophila melanogaster* shall be conducted for ODA if it produces a positive result in the detection of gene mutation assay in somatic cells in culture conducted pursuant to paragraph (c)(3)(i)(A) of this section.

(C) An oral mouse visible specific locus test shall be conducted for ODA if it produces a positive result in the sex linked recessive lethal test in *Drosophila melanogaster* conducted pursuant to paragraph (c)(3)(i)(B) of this section and if so required in a FEDERAL REGISTER notice or certified letter sent to test sponsors.

(ii) *Test standard.* (A) (*1*) The detection of gene mutations in somatic cells in culture shall be conducted with ODA in accordance with § 798.5300 of this chapter, except for the provisions of paragraphs (d)(3) (i), (ii) and (4) of § 798.5300.

(*2*) For purposes of this section, the following provisions also apply:

(*i*) *Types of cells used in the assay.* ODA shall be tested in L5178Y mouse lymphoma cells. Cells should be checked for *Mycoplasma* contamination and may be periodically checked for karyotype stability.

(*ii*) *Cell growth and maintenance.* Alternative dosing procedures consisting of suspension cultures or roller-bottle incubation shall be used. Appropriate incubation conditions ($CO_2$ concentrations, temperature, and humidity) shall be used.

(*iii*) *Metabolic activation.* The metabolic activation system shall be derived from the postmitochondrial fraction (S-9) of livers from rats pretreated with Aroclor 1254. Cells shall be exposed to test substance both in the presence and absence of an appropriate metabolic activation system.

(B) (*1*) The sex-linked recessive lethal test in *Drosophila melanogaster* shall be conducted with ODA in accordance with § 798.5275 of this chapter except for the provisions of paragraph (d)(5)(iii) of § 798.5275.

(*2*) For purposes of this section, the following provisions also apply:

(*i*) *Route of administration.* The route of administration shall be oral.

(*ii*) [Reserved]

(*iii*) *Reporting requirements.* (A) Gene mutation tests shall be completed and the final reports submitted to EPA as follows:

(*1*) The detection of gene mutations in somatic cells in culture shall be completed within 10 months of the date specified in paragraph (d)(1) of this section.

(*2*) The sex-linked recessive lethal test in *Drosophila melanogaster* (if required) shall be completed within 22 months of the date specified in paragraph (d)(1) of this section.

(B) Interim progress reports shall be provided to EPA at 6-month intervals for each test beginning 6 months after the date specified in paragraph (d)(1) of this section until submission of the final report.

(4) *Oncogenicity*—(i) *Required testing.* An oncogenicity bioassay shall be conducted orally for ODA if positive

results occur in any of the following tests and if so required in a Federal Register notice or certified letter sent to test sponsors.

(A) *In vivo* mammalian bone marrow cytogenetics tests conducted pursuant to paragraph (c)(2)(i)(A) of this section.

(B) Detection of gene mutation in somatic cells in culture assay conducted pursuant to paragraph (c)(3)(i)(A) of this section.

(C) Sex linked recessive lethal test in *Drosophila melanogaster,* conducted pursuant to paragraph (c)(3)(i)(B) of this section.

(ii) *Test standard.* (A)(*1*) The oncogenicity bioassay shall be conducted with ODA in accordance with § 798.3300 of this chapter, except for the provisions of paragraphs (b)(1)(i) and (6) of § 798.3300.

(*2*) For purposes of this section, the following provisions also apply:

(*i*) *Species and strain.* ODA shall be tested in both rats and mice. Commonly used laboratory strains shall be employed.

(*ii*) *Administration of the test substance.* The route of administration shall be oral by gavage.

(iii) *Reporting requirements.* (A) The oncogenicity bioassay shall be completed and the final report submitted to EPA within 53 months of EPA's notification of the test sponsor by certified letter or Federal Register notice under paragraph (c)(4)(i) of this section that testing should be initiated.

(B) Interim progress reports shall be provided at 6-month intervals beginning 6 months after the notification under paragraph (c)(4)(i) of this section until submission of the final report.

(d) Effective dates. (1) Section 799.3175 is effective October 7, 1987 except for paragraphs (c)(1) (ii) and (iii); (2) (ii) and (iii); (3) (ii) and (iii); (4) (ii) and (iii), and (d) which are effective on January 17, 1989.

(2) The guidelines and other test methods cited in this section are referenced here as they exist on January 17, 1989.

(Information collection requirements approved by the Office of Management and Budget under control number 2070-0033)

[52 FR 31969, Aug. 24, 1987, as amended at 53 FR 48546, Dec. 1, 1988]

### § 799.3450 Propylene oxide.

(a) *Identification of test substance.* (1) Propylene oxide (CAS No. 75-56-9) shall be tested in accordance with this section.

(2) Propylene oxide of at least 99.0-percent purity shall be used as the test substance in all tests.

(b) *Persons required to submit study plans, conduct tests, and submit data.* (1) All persons who manufacture or process propylene oxide, other than as an inpurity, from January 10, 1986, to the end of the reimbursement period shall submit letters of intent to conduct testing or exemption applications, study plans, and shall conduct tests, and submit data as specified in this section, Subpart A of this part, and Part 790 of this chapter.

(2) Persons subject to this section are not subject to the requirements of § 790.50(a)(2), (5), and (6) and (b) and § 790.87(a)(1)(ii) of this chapter.

(3) Persons who notify EPA of their intent to conduct tests in compliance with the requirements of this section must submit plans for those tests no later than 30 days before the initiation of each of those tests.

(4) In addition to the requirements of § 790.87(a)(2) and (3) of this chapter, EPA will conditionally approve exemption applications for this rule if EPA has received a letter of intent to conduct the testing from which exemption is sought and EPA has adopted test standards and schedules in a final Phase II test rule.

(c) *Health effects testing*—(1) Developmental toxicity—(i) *Required testing.* An inhalation developmental toxicity test in the rat shall be conducted with propylene oxide.

(ii) *Test standards.* The inhalation developmental toxicity testing shall be conducted in accordance with the EPA-approved study plans (July 22, 1987): "Range-finding Inhalation Developmental Toxicity Study in Rats" and "Inhalation Developmental Toxicity Study in Rats". Copies of these EPA-approved study plans are located in the rulemaking record for this rule (docket no. OPTS-42028D) and are available for inspection in EPA's

OPTS Reading Room, NE-G004, 401 M Street SW., Washington, DC 20460, from 8 a.m. to 4 p.m., Monday through Friday, except legal holidays.

(iii) *Reporting requirements.* (A) The developmental toxicity tests shall be completed and the final reports submitted to EPA within 12 months of the effective date of the final Phase II rule.

(B) An interim progress report shall be submitted to EPA 6 months after the effective date of the final Phase II rule.

(2) [Reserved]

(d) *Effective date.* The effective date of the final Phase II rule requiring inhalation developmental toxicity testing of propylene oxide is November 6, 1987.

(Information collection requirements approved by the Office of Management and Budget under control number 2070-0033)

[50 FR 48770, Nov. 27, 1985, as amended at 52 FR 35709, Sept. 23, 1987]

### § 799.4000 Tetrabromobisphenol A.

(a) *Identification of test substance.* (1) Tetrabromobisphenol A (TBBPA, CAS No. 79-94-7) shall be tested in accordance with this section.

(2) Tetrabromobisphenol A of at least 98 percent purity shall be used as the test substance.

(b) *Persons required to submit study plans, conduct tests, and submit data.* All persons who manufacture (including import) or process or intend to manufacture or process tetrabromobisphenol A, other than as an impurity, after August 19, 1987, to the end of the reimbursement period shall submit letters of intent to conduct testing, submit study plans, conduct tests, and submit data or submit exemption applications as specified in this section, Subpart A of this part, and Parts 790 and 792 of this chapter for single-phase rulemaking.

(c) *Chemical fate*—(1) *Biodegradability in sediment/water*—(i) *Required testing.* Biodegradation testing in sediment/water shall be conducted with TBBPA using clean, freshwater sediments in accordance with the method described in an A.W. Bourquin article entitled "An Artificial Microbial Ecosystem for Determining Effects and Fate of Toxicants in a Salt-Marsh Environment", published in *Developments in Industrial Microbiology,* Vol. 18, Chapter 11, 1977, which is incorporated by reference. The method is available from the Office of the Federal Register Information Center, 11th and L St., NW., Washington, DC, 20408, and in the EPA OPTS Reading Room, Rm. G-004 Northeast Mall, 401 M St., SW., Washington, DC 20460. This incorporation by reference was approved by the Director of the Federal Register in accordance with 5 U.S.C. 552(a) and 1 CFR Part 51. The method is incorporated as it exists on the effective date of the final rule and a notice of any change to the method will be published in the FEDERAL REGISTER.

(ii) *Reporting requirements.* (A) The biodegradation test in sediment/water shall be completed and the final report submitted to EPA within 18 months of the effective date of the final rule.

(B) An interim progress report shall be submitted to EPA 6 months after the effective date of the final rule.

(2) *Inherent biodegradability in soil*—(i) *Required testing.* Inherent biodegradability in soil tests to assess aerobic and anaerobic biodegradability shall be conducted with TBBPA in accordance with § 796.3400 of this chapter.

(ii) *Reporting requirements.* (A) The inherent biodegradability in soil tests shall be completed and the final reports submitted to EPA within 18 months of the effective date of the final rule.

(B) An interim progress report shall be submitted to EPA 6 months after the effective date of the final rule.

(d) *Environmental effects*—(1) *Algal acute toxicity*—(i) *Required testing.* Algal acute toxicity testing shall be conducted with TBBPA using *Selenastrum capricornutum* in accordance with § 797.1050 of this chapter.

(ii) *Reporting requirements.* (A) The algal acute toxicity test shall be completed and the final report submitted to EPA within 15 months of the effective date of the final rule.

(B) An interim progress report shall be submitted to EPA 6 months after the effective date of the final rule.

(2) *Fish acute toxicity*—(i) *Required testing.* Fish acute toxicity testing shall be conducted with TBBPA using *Pimephales promelas* (fathead minnow) in accordance with § 797.1400 of this chapter.

(ii) *Reporting requirements.* (A) The fish acute toxicity test shall be completed and the final report submitted to EPA within 15 months of the effective date of the final rule.

(B) An interim progress report shall be submitted to EPA 6 months after the effective date of the final rule.

(3) *Midge partial life-cycle toxicity in sediments*—(i) *Required testing.* A 14-day toxicity test in a flow-through system shall be conducted with the midge (*Chironomus tentans*) using TBBPA-spiked clean, freshwater sediments having low, medium, and high organic carbon content in accordance with the American Society for Testing and Materials Special Technical Publication 854 (ASTM STP 854), entitled "Aquatic Safety Assessment of Chemicals Sorbed to Sediments," by W.J. Adams et. al., and published in *Aquatic Toxicology and Hazard Assessment: Seventh Symposium*, ASTM STP 854, pp. 429-453, R.D. Cardwell et. al., Eds. 1985, which is incorporated by reference. The method is available from the Office of the Federal Register Information Center, 11th and L St., NW., Washington, DC, 20408, and in the EPA OPTS Reading Room, Rm G-004 Northeast Mall, 401 M St., SW., Washington, DC 20460. This incorporation by reference was approved by the Director of the Federal Register in accordance with 5 U.S.C. 552(a) and 1 CFR Part 51. The method is incorporated as it exists on the effective date of this rule and a notice of any change to the method will be published in the FEDERAL REGISTER.

(ii) *Reporting requirements.* (A) The 14-day toxicity test with midge using sediments shall be conducted and the final report submitted to EPA within 2 years of the effective date of the final rule.

(B) Interim progress reports shall be submitted to EPA at 6-month intervals beginning 6 months after the effective date of the final rule, until the final report is submitted to EPA.

(4) *Daphnid chronic toxicity*—(i) *Required testing.* Daphnid chronic toxicity testing shall be conducted with TBBPA using *Daphnia magna* or *D. pulex* in accordance with § 797.1330 of this chapter.

(ii) *Reporting requirements.* (A) The daphnid chronic toxicity test shall be completed and the final report submitted to EPA within 2 years of the effective date of the final rule.

(B) Interim progress reports shall be submitted to EPA at 6-month intervals beginning 6 months after the effective date of the final rule, until the final report is submitted to EPA.

(5) *Fish early life stage toxicity*—(i) *Required testing.* (A) A fish early life stage toxicity test shall be conducted with TBBPA. The test species shall be fathead minnow (*Pimephales promelas*) if the 96-hour $LC_{50}$ for fathead minnow conducted in accordance with paragraph (d)(2) of this section is equal to or less than 0.8 mg/L; the test species shall be either fathead minnow or rainbow trout if the 96-hour $LC_{50}$ for fathead minnow is between 0.08-2.0 mg/L; the test species shall be rainbow trout if the 96-hour $LC_{50}$ for fathead minnow is greater than or equal to 2.0 mg/L. The fish early life stage toxicity test shall be conducted in accordance with § 797.1600 of this chapter, except for the provisions in paragraphs (c)(4)(iv)(A), (d)(2)(vii)(A)(*2*), (3)(i) and (ii)(B)(*1*), and (iv)(A) of § 797.1600.

(B) For the purpose of this section, the following provisions also apply:

(*1*) The first feeding for the fathead and sheepshead minnow fry shall begin shortly after transfer of the fry from the embryo cups to the test chambers. Silversides are fed the first day after hatch. Trout species initiate feeding at swim-up. The trout fry shall be fed trout starter mash or live newly-hatched brine shrimp nauplii (*Artemia salina*) three times a day *ad libitum*, with excess food siphoned off daily. The minnow fry shall be fed *Artemia salina* at least three times a day.

(*2*) The concentration of dissolved oxygen in the dilution water (fresh or salt) shall be greater than 75 percent of air saturation. When necessary, dilution water should be aerated by means of airstones, surface aerators, or screen tubes before the introduction of the test substance.

(*3*) Dissolved oxygen concentration. It is recommended that the dissolved oxygen concentration be maintained between 90 and 100 percent saturation; but it shall be no less than 75 percent saturation at all times for both minnow species, silversides, and the trout species in all test chambers. Dilution water in the head box may be aerated, but the test solution itself shall not be aerated.

(*4*) The concentration of dissolved oxygen shall not fall below 75 percent saturation for the fathead and sheepshead minnows and for the rainbow and brook trout.

(5) Brook and rainbow trout embryos shall be maintained in darkness or very low light intensity through 1-week post-hatch, at which time a 16-hour light and 8-hour dark photoperiod shall be provided.

(6) *Bioconcentration in fish*—(i) *Required testing.* A bioconcentration test shall be conducted with TBBPA using *Pimephales promelas* (fathead minnow) in accordance with § 797.1520 of this chapter.

(ii) *Reporting requirements.* (A) The bioconcentration test in fish shall be completed and the final report submitted to EPA within 18 months after the effective date of the final rule.

(B) An interim progress report shall be submitted to EPA 6-months after the effective date of the final rule.

(7) *Bioconcentration in oyster*—(i) *Required testing.* A bioconcentration test shall be conducted with TBBPA using *Crassostrea virginica* (oyster) in accordance with § 797.1830 of this chapter.

(ii) *Reporting requirements.* (A) The bioconcentration test in oyster shall be completed and the final report submitted to EPA within 18 months of the effective date of the final rule.

(B) Interim progress reports shall be submitted to EPA at 6-month intervals beginning 6 months after the effective date of the final rule, until the final report is submitted to EPA.

(e) *Effective date.* The effective date of the final rule is August 19, 1987.

(Information collection requirements have been approved by the Office of Management and Budget under control number 2070-0033)

[52 FR 25225, July 6, 1987, as amended at 54 FR 27358, June 29, 1989]

### § 799.4400 1,1,1-Trichloroethane.

(a) *Identification of chemical test substance.* 1,1,1-Trichloroethane (CAS No. 71-55-6, also known as methyl chloroform) shall be tested in accordance with this part.

(b) *Identification of test substance.* 1,1,1-Trichloroethane stabilized with less than 0.1 percent butylene oxide shall be used as the test substance in all tests.

(c) *Persons required to submit study plans, conduct tests and submit data.* All persons who manufacture or process 1,1,1-trichloroethane, other than as an impurity, from November 23, 1984, to the end of the reimbursement period shall submit letters of intent to test, exemption applications, and study plans and shall conduct tests and submit data as specified in this section, Subpart A of this part and Part 790 of this chapter (Test Rule Development and Exemption Procedures).

(d) *Health effects testing*—(1) Developmental toxicity—(i) *Required testing.* A test for developmental toxicity shall be conducted with 1,1,1-trichloroethane.

(ii) *Testing standards.* The testing shall be conducted in accordance with the following study plans developed by the Halogenated Solvents Industry Alliance (HSIA), 1612 K St., NW., Washington, DC 20006, and submitted to the Agency on February 21, 1985 and April 17, 1985: Inhalation Developmental Toxicity Probe Study in Rats, Inhalation Developmental Toxicity in Rats, Inhalation Developmental Probe Study in Rabbits, and Inhalation Development Toxicity Study in Rabbits, which are incorporated by reference. Copies of these study plans are located in the public record for this rule (Docket No. OPTS-42059B) and are available for inspection at the Office of the Federal Register, Rm. 8401, 1100 L St., NW., Washington, DC. These incorporations by reference were approved by the Director of the Federal Register in January 1985.

These materials are incorporated as they exist on the date of the approval, and a notice of any change in these materials will be published in the FEDERAL REGISTER. Copies of the incorporated material may be obtained from the TSCA Public Docket Office (TS-793), Rm. NE-G004, Office of Toxic Substances, Environmental Protection Agency, 401 M St., SW., Washington, DC 20460.

(iii) *Reporting requirements.* (A) The developmental toxicity testing shall be initiated within 6 months of the effective date of the final Phase II rule.

(B) The developmental toxicity tests shall be completed and the final results submitted to the Agency within 16 months of the effective date of the final Phase II rule.

(C) Progress reports shall be submitted quarterly to the Agency beginning 90 days from the effective date of the final Phase II rule.

(2) [Reserved]

(Information collection requirements approved by the Office of Management and Budget under control number 2070-0033)

[49 FR 39817, Oct. 10, 1984, as amended at 50 FR 51685, Dec. 19, 1985; 53 FR 12526, Apr. 15, 1988]

### § 799.4440 Triethylene glycol monomethyl ether.

(a) *Identification of test substance.* (1) Triethylene glycol monomethyl ether (TGME, CAS No. 112-35-6) shall be tested in accordance with this section.

(2) TGME of at least 90 percent purity shall be used as the test substance.

(b) *Persons required to submit study plans, conduct tests, and submit data.* All persons who manufacture or process TGME, other than as an impurity, after May 17, 1989, to the end of the reimbursement period shall submit letters of intent to conduct testing, submit study plans, conduct tests and submit data, or submit exemption applications as specified in this section, Subpart A of this part, and Parts 790 and 792 of this chapter for single-phase rulemaking.

(c) *Developmental neurotoxicity*—(1) *Required testing.* Developmental neurotoxicity testing shall be performed in the Sprague-Dawley rat by gavage in accordance with § 795.250 of this chapter except for the provision in paragraph (c)(3)(iii) of § 795.250.

(2) For the purpose of this section, the following provision also applies:

(i) *Dose levels and dose selection.* In the absence of developmental toxicity or maternal toxicity the maximum dose shall be 5 grams/kilogram.

(ii) [*Reserved*]

(3) *Reporting requirements*—(i) The developmental neurotoxicity test shall be completed and the final report submitted to EPA within 21 months of the initiation of the test. The test shall be initiated within 44 days of the publication in the FEDERAL REGISTER of notice of EPA's receipt of TGME developmental toxicity data.

(ii) Progress reports shall be submitted to EPA at 6- month intervals, beginning six months after the initiation of the test.

(d) *Effective date.* (1) The effective date of the final rule is May 17, 1989.

(2) The guideline cited in this section is referenced here as it exists on May 17, 1989.

(Information collection requirements have been approved by the Office of Management and Budget under control number 2070-0033)

[54 FR 13477, Apr. 3, 1989]

## Subpart C—Testing Consent Orders

### § 799.5000 Testing consent orders.

This section sets forth a list of substances and mixtures which are the subject of testing consent orders adopted under 40 CFR Part 790. Listed below in Chemical Abstract Service (CAS) Registry Number order are the substances and mixtures which are the subject of these orders and the FEDERAL REGISTER citations providing public notice of such orders.

| CAS Number | Substance or mixture name | Testing | FR citation |
|---|---|---|---|
| 62-53-3 | Aniline | Health effects | August 19, 1988. |
| | Environmental effects. | Do. | |
| 84-74-2 | Di-n-butyl phthalate. | Environmental effects. | January 9, 1989. |
| 84-75-3 | Di-n-hexyl phthalate. | Environmental effects. | January 9, 1989. |
| | | Chemical fate | Do. |

| CAS Number | Substance or mixture name | Testing | FR citation |
|---|---|---|---|
| 88-74-4 2 | Nitroaniline | Health effects | August 19, 1988. |
| 95-51-2 2 | Chloroaniline | Health effects<br>Environmental effects. | Do.<br>Do. |
| 95-76-1 3,4 | Dichloroaniline | Health effects | Do. |
| 97-02-9 2,4 | Dinitroaniline | Health effects | Do. |
| 99-30-9 2,6 | Dichloro-4-nitro-aniline. | Environmental effects. | Do. |
| 100-01-6 4 | Nitroaniline | Health effects | Do. |
| 106-47-8 4 | Chloroaniline | Health effects | Do. |
| 112-35-6 | Triethylene glycol monomethyl ether. | Health effects | April 3, 1989. |
| 112-50-5 | Triethylene glycol monoethyl ether. | Health effects | Do. |
| 117-81-7 | Di-2-ethylhexyl phthalate. | Chemical fate | January 9, 1989. |
| 119-06-2 | Ditridecyl phthalate. | Chemical fate | Do. |
| 131-11-3 | Dimethly phthalate. | Environmental effects. | Do. |
| 143-22-6 | Triethylene glycol monobutyl ether. | Health effects | Do. |
| 328-84-7 | 3,4-Dichlorobenzotrifluoride. | Environmental effects.<br>Chemical fate | June 23, 1987.<br>June 23, 1987. |
| 556-67-2 | Octamethylcyclotetrasiloxane. | Chemical fate<br>Environmental effects. | January 10, 1989.<br>Do. |
| 1634-04-4 | Methyl tert-butyl ether. | Health effects | March 31, 1988. |
| 3648-20-2 | Diundecyl phthalate. | Environmental effects. | January 9, 1989. |
| 25550-98-5 | Diisodecyl phenyl phosphite. | Neurotoxic effects. | February 24, 1989. |
| 26761-40-0 | Diisodecyl phthalate. | Chemical fate | January 9, 1989. |
| 68515-42-4 | Di (heptyl, nonyl, undecyl phthalate (mixed isomers). | Environmental effects. | Do |
| 68515-47-9 | Ditridecyl phthalate (mixed isomers). | Chemical fate | Do. |
| 68515-49-1 | Diisodecyl phthalate (mixed isomers). | Chemical fate | Do. |
| 68515-50-4 | Dihexyl phthalate (mixed isomers). | Environmental effects.<br>Chemical fate | Do.<br>Do. |

[52 FR 23548, June 23, 1987, as amended at 53 FR 10394, Mar. 31, 1988; 53 FR 31813, Aug. 19, 1988; 53 FR 49966, Dec. 12, 1988; 54 FR 620, Jan. 9, 1989; 54 FR 821, Jan. 10, 1989; 54 FR 3183, Jan. 23, 1989; 54 FR 8114, Feb. 24, 1989; 54 FR 13472, Apr. 3, 1989]

## Subpart D—Multichemical Test Rules

### § 799.5055 Hazardous waste constituents subject to testing.

(a) *Identification of test substances.* (1) The table in paragraph (c) of this section identifies those chemical substances that shall be tested in accordance with this section.

(2) Substances of at least 98-percent purity shall be used as the test substances.

(b) *Persons required to submit study plans, conduct tests, and submit data.* All persons who manufacuture (including import or manufacture as a byproduct) or process or intend to manufacture or process one or more of the substances in paragraph (c) of this section, other than as an impurity, after July 29, 1988, to the end of the reimbursement period shall submit letters of intent to conduct testing, submit study plans, conduct tests, and submit data, or submit exemption applications for those substances they manufacture or process, or intend to manufacture or process, as specified in this section, Subpart A of this part, and Parts 790 and 792 of this chapter for single-phase rulemaking.

(c) *Designation of testing.* The substances identified in the following table by name and CAS number shall be tested in accordance with the designated requirements under paragraphs (d) and (e) of this section. The paragraph numbers listed for a substance refer to the specific testing and reporting requirements specified in paragraphs (d) and (e) of this section.

| Chemical name | CAS No. | Required testing under paragraphs (d) and (e) of this section |
|---|---|---|
| Acetamide, 2-fluoro | 640-19-7 | (e)(1) |
| Bis(2-chloroethoxy)methane. | 111-91-1 | (d)(2), (e)(1) |
| Bis(2-chloroisopropyl)ether. | 108-60-1 | (d)(2) |
| 4-Bromobenzyl cyanide | 16532-79-9 | (d)(1), (2), (e)(1) |
| Bromoform | 75-25-2 | (d)(2) |
| 4-Chlorobenzo-trichloride | 5216-25-1 | (e)(1) |
| 2,4-D | 94-75-7 | (d)(2) |

| Chemical name | CAS No. | Required testing under paragraphs (d) and (e) of this section |
|---|---|---|
| Dibromomethane 74-95-3 (d)(2). | | |
| 1,2-Dichlorobenzene | 95-50-1 | (d)(2) |
| 1,1-Dichloroethane | 75-34-3 | (d)(2) |
| 1,3-Dichloropropanol | 96-23-1 | (d)(1), (e)(1) |
| Dihydrosafrole | 94-58-6 | (d)(2) |
| Endrin | 72-20-8 | (d)(2) |
| Ethyl methacrylate | 97-63-2 | (d)(2) |
| Maleic hydrazide | 123-33-1 | (d)(1), (2) |
| Malononitrile | 109-77-3 | (d)(1), (e)(1) |
| Methanethiol | 74-93-1 | (d)(1) |
| Methyl chloride | 74-87-3 | (d)(2) |
| *p*-Nitrophenol | 100-02-7 | (e)(1) |
| Pentachlorobenzene | 608-93-5 | (d)(2) |
| Pentachloroethane | 76-01-7 | (d)(2) |
| 1,2,4,5-Tetrachlorobenzene. | 95-94-3 | (d)(2) |
| Trichloromethanethiol | 594-42-3 | (d)(1), (2), (e)(1) |

(d) *Chemical fate testing*—(1) *Soil adsorption*—(i) *Required testing.* A soil adsorption isotherm test shall be conducted with the substances designated in paragraph (c) of this section in accordance with § 796.2750 of this chapter.

(ii) *Reporting requirements.* The sediment and soil adsorption isotherm tests shall be completed and the final results submitted to the Agency within 9 months of the effective date of the final rule.

(2) *Hydrolysis*—(i) *Required testing.* A test of hydrolysis as a function of pH at 25 °C shall be conducted with the substances designated in paragraph (c) of this section in accordance with § 796.3500 of this chapter.

(ii) *Reporting requirement.* The hydrolysis tests shall be completed and the final results submitted to the Agency within 6 months of the effective date of the final rule.

(e) *Health effects testing*—(1) *Subchronic toxicity*—(i) *Required testing.* An oral gavage subchronic toxicity test shall be conducted in the rat with the substances designated in paragraph (c) of this section in accordance with § 798.2650 of this chapter.

(ii) *Reporting requirements.* (A) The oral gavage subchronic tests shall be completed and the final results submitted to the Agency within 1 year of the effective date of the final rule.

(B) Progress reports for each test shall be submitted to the Agency 6 months after the effective date of the final rule.

(2) [Reserved]

(f) *Effective date.* (1) The effective date of the final rule July 29, 1988.

(2) The guidelines and other test methods cited in this section are referenced here as they exist on June 15, 1988.

(The information collection requirements have been approved by the Office of Management and Budget under control number 2070-0033)

[53 FR 22324, June 15, 1988; 53 FR 48645, Dec. 2, 1988]

# CHAPTER V—COUNCIL ON ENVIRONMENTAL QUALITY

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## PART 1500—PURPOSE, POLICY, AND MANDATE

Sec.
1500.1 Purpose.
1500.2 Policy.
1500.3 Mandate.
1500.4 Reducing paperwork.
1500.5 Reducing delay.
1500.6 Agency authority.

AUTHORITY: NEPA, the Environmental Quality Improvement Act of 1970, as amended (42 U.S.C. 4371 *et seq.*), sec. 309 of the Clean Air Act, as amended (42 U.S.C. 7609) and E.O. 11514, Mar. 5, 1970, as amended by E.O. 11991, May 24, 1977).

SOURCE: 43 FR 55990, Nov. 28, 1978, unless otherwise noted.

### § 1500.1 Purpose.

(a) The National Environmental Policy Act (NEPA) is our basic national charter for protection of the environment. It establishes policy, sets goals (section 101), and provides means (section 102) for carrying out the policy. Section 102(2) contains "action-forcing" provisions to make sure that federal agencies act according to the letter and spirit of the Act. The regulations that follow implement section 102(2). Their purpose is to tell federal agencies what they must do to comply with the procedures and achieve the goals of the Act. The President, the federal agencies, and the courts share responsibility for enforcing the Act so as to achieve the substantive requirements of section 101.

(b) NEPA procedures must insure that environmental information is available to public officials and citizens before decisions are made and before actions are taken. The information must be of high quality. Accurate scientific analysis, expert agency comments, and public scrutiny are essential to implementing NEPA. Most important, NEPA documents must concentrate on the issues that are truly significant to the action in question, rather than amassing needless detail.

(c) Ultimately, of course, it is not better documents but better decisions that count. NEPA's purpose is not to generate paperwork—even excellent paperwork—but to foster excellent action. The NEPA process is intended to help public officials make decisions that are based on understanding of environmental consequences, and take actions that protect, restore, and enhance the environment. These regulations provide the direction to achieve this purpose.

### § 1500.2 Policy.

Federal agencies shall to the fullest extent possible:

(a) Interpret and administer the policies, regulations, and public laws of the United States in accordance with the policies set forth in the Act and in these regulations.

(b) Implement procedures to make the NEPA process more useful to decisionmakers and the public; to reduce paperwork and the accumulation of extraneous background data; and to emphasize real environmental issues and alternatives. Environmental impact statements shall be concise, clear, and to the point, and shall be supported by evidence that agencies have made the necessary environmental analyses.

(c) Integrate the requirements of NEPA with other planning and environmental review procedures required by law or by agency practice so that all such procedures run concurrently rather than consecutively.

(d) Encourage and facilitate public involvement in decisions which affect the quality of the human environment.

(e) Use the NEPA process to identify and assess the reasonable alternatives to proposed actions that will avoid or minimize adverse effects of these actions upon the quality of the human environment.

(f) Use all practicable means, consistent with the requirements of the Act and other essential considerations of national policy, to restore and enhance the quality of the human environment and avoid or minimize any possible adverse effects of their actions upon the quality of the human environment.

### § 1500.3 Mandate.

Parts 1500 through 1508 of this title provide regulations applicable to and binding on all Federal agencies for implementing the procedural provisions of the National Environmental Policy Act of 1969, as amended (Pub. L. 91-190, 42 U.S.C. 4321 et seq.) (NEPA or the Act) except where compliance would be inconsistent with other statutory requirements. These regulations are issued pursuant to NEPA, the Environmental Quality Improvement Act of 1970, as amended (42 U.S.C. 4371 et seq.) section 309 of the Clean Air Act, as amended (42 U.S.C. 7609) and Executive Order 11514, Protection and Enhancement of Environmental Quality (March 5, 1970, as amended by Executive Order 11991, May 24, 1977). These regulations, unlike the predecessor guidelines, are not confined to sec. 102(2)(C) (environmental impact statements). The regulations apply to the whole of section 102(2). The provisions of the Act and of these regulations must be read together as a whole in order to comply with the spirit and letter of the law. It is the Council's intention that judicial review of agency compliance with these regulations not occur before an agency has filed the final environmental impact statement, or has made a final finding of no significant impact (when such a finding will result in action affecting the environment), or takes action that will result in irreparable injury. Furthermore, it is the Council's intention that any trivial violation of these regulations not give rise to any independent cause of action.

### § 1500.4 Reducing paperwork.

Agencies shall reduce excessive paperwork by:

(a) Reducing the length of environmental impact statements (§ 1502.2(c)), by means such as setting appropriate page limits (§§ 1501.7(b)(1) and 1502.7).

(b) Preparing analytic rather than encyclopedic environmental impact statements (§ 1502.2(a)).

(c) Discussing only briefly issues other than significant ones (§ 1502.2(b)).

(d) Writing environmental impact statements in plain language (§ 1502.8).

(e) Following a clear format for environmental impact statements (§ 1502.10).

(f) Emphasizing the portions of the environmental impact statement that are useful to decisionmakers and the public (§§ 1502.14 and 1502.15) and reducing emphasis on background material (§ 1502.16).

(g) Using the scoping process, not only to identify significant environmental issues deserving of study, but also to deemphasize insignificant issues, narrowing the scope of the environmental impact statement process accordingly (§ 1501.7).

(h) Summarizing the environmental impact statement (§ 1502.12) and circulating the summary instead of the entire environmental impact statement if the latter is unusually long (§ 1502.19).

(i) Using program, policy, or plan environmental impact statements and tiering from statements of broad scope to those of narrower scope, to eliminate repetitive discussions of the same issues (§§ 1502.4 and 1502.20).

(j) Incorporating by reference (§ 1502.21).

(k) Integrating NEPA requirements with other environmental review and consultation requirements (§ 1502.25).

(l) Requiring comments to be as specific as possible (§ 1503.3).

(m) Attaching and circulating only changes to the draft environmental impact statement, rather than rewriting and circulating the entire statement when changes are minor (§ 1503.4(c)).

(n) Eliminating duplication with State and local procedures, by providing for joint preparation (§ 1506.2), and with other Federal procedures, by providing that an agency may adopt appropriate environmental documents prepared by another agency (§ 1506.3).

(o) Combining environmental documents with other documents (§ 1506.4).

(p) Using categorical exclusions to define categories of actions which do not individually or cumulatively have a significant effect on the human environment and which are therefore exempt from requirements to prepare an environmental impact statement (§ 1508.4).

(q) Using a finding of no significant impact when an action not otherwise excluded will not have a significant effect on the human environment and is therefore exempt from requirements to prepare an environmental impact statement (§ 1508.13).

[43 FR 55990, Nov. 29, 1978; 44 FR 873, Jan. 3, 1979]

### § 1500.5 Reducing delay.

Agencies shall reduce delay by:

(a) Integrating the NEPA process into early planning (§ 1501.2).

(b) Emphasizing interagency cooperation before the environmental impact statement is prepared, rather than submission of adversary comments on a completed document (§ 1501.6).

(c) Insuring the swift and fair resolution of lead agency disputes (§ 1501.5).

(d) Using the scoping process for an early identification of what are and what are not the real issues (§ 1501.7).

(e) Establishing appropriate time limits for the environmental impact statement process (§§ 1501.7(b)(2) and 1501.8).

(f) Preparing environmental impact statements early in the process (§ 1502.5).

(g) Integrating NEPA requirements with other environmental review and consultation requirements (§ 1502.25).

(h) Eliminating duplication with State and local procedures by providing for joint preparation (§ 1506.2) and with other Federal procedures by providing that an agency may adopt appropriate environmental documents prepared by another agency (§ 1506.3).

(i) Combining environmental documents with other documents (§ 1506.4).

(j) Using accelerated procedures for proposals for legislation (§ 1506.8).

(k) Using categorical exclusions to define categories of actions which do not individually or cumulatively have a significant effect on the human environment (§ 1508.4) and which are therefore exempt from requirements to prepare an environmental impact statement.

(l) Using a finding of no significant impact when an action not otherwise excluded will not have a significant effect on the human environment (§ 1508.13) and is therefore exempt from requirements to prepare an environmental impact statement.

### § 1500.6 Agency authority.

Each agency shall interpret the provisions of the Act as a supplement to its existing authority and as a mandate to view traditional policies and missions in the light of the Act's national environmental objectives. Agencies shall review their policies, procedures, and regulations accordingly and revise them as necessary to insure full compliance with the purposes and provisions of the Act. The phrase "to the fullest extent possible" in section 102 means that each agency of the Federal Government shall comply with that section unless existing law applicable to the agency's operations expressly prohibits or makes compliance impossible.

## PART 1501—NEPA AND AGENCY PLANNING

Sec.

1501.1 Purpose.
1501.2 Apply NEPA early in the process.
1501.3 When to prepare an environmental assessment.
1501.4 Whether to prepare an environmental impact statement.
1501.5 Lead agencies.
1501.6 Cooperating agencies.
1501.7 Scoping.
1501.8 Time limits.

AUTHORITY: NEPA, the Environmental Quality Improvement Act of 1970, as amended (42 U.S.C. 4371 *et seq.*), sec. 309 of the Clean Air Act, as amended (42 U.S.C. 7609, and E.O. 11514 (Mar. 5, 1970, as amended by E.O. 11991, May 24, 1977).

SOURCE: 43 FR 55992, Nov. 29, 1978, unless otherwise noted.

### § 1501.1 Purpose.

The purposes of this part include:

(a) Integrating the NEPA process into early planning to insure appropriate consideration of NEPA's policies and to eliminate delay.

(b) Emphasizing cooperative consultation among agencies before the environmental impact statement is prepared rather than submission of adversary comments on a completed document.

(c) Providing for the swift and fair resolution of lead agency disputes.

(d) Identifying at an early stage the significant environmental issues deserving of study and deemphasizing insignificant issues, narrowing the scope of the environmental impact statement accordingly.

(e) Providing a mechanism for putting appropriate time limits on the environmental impact statement process.

### § 1501.2 Apply NEPA early in the process.

Agencies shall integrate the NEPA process with other planning at the earliest possible time to insure that planning and decisions reflect environmental values, to avoid delays later in the process, and to head off potential conflicts. Each agency shall:

(a) Comply with the mandate of section 102(2)(A) to "utilize a systematic, interdisciplinary approach which will insure the integrated use of the natural and social sciences and the environmental design arts in planning and in decisionmaking which may have an impact on man's environment," as specified by § 1507.2.

(b) Identify environmental effects and values in adequate detail so they can be compared to economic and technical analyses. Environmental documents and appropriate analyses shall be circulated and reviewed at the same time as other planning documents.

(c) Study, develop, and describe appropriate alternatives to recommended courses of action in any proposal which involves unresolved conflicts concerning alternative uses of available resources as provided by section 102(2)(E) of the Act.

(d) Provide for cases where actions are planned by private applicants or other non-Federal entities before Federal involvement so that:

(1) Policies or designated staff are available to advise potential applicants of studies or other information foreseeably required for later Federal action.

(2) The Federal agency consults early with appropriate State and local agencies and Indian tribes and with interested private persons and organizations when its own involvement is reasonably foreseeable.

(3) The Federal agency commences its NEPA process at the earliest possible time.

### § 1501.3 When to prepare an environmental assessment.

(a) Agencies shall prepare an environmental assessment (§ 1508.9) when necessary under the procedures adopted by individual agencies to supplement these regulations as described in § 1507.3. An assessment is not necessary if the agency has decided to prepare an environmental impact statement.

(b) Agencies may prepare an environmental assessment on any action at any time in order to assist agency planning and decisionmaking.

### § 1501.4 Whether to prepare an environmental impact statement.

In determining whether to prepare an environmental impact statement the Federal agency shall:

(a) Determine under its procedures supplementing these regulations (described in § 1507.3) whether the proposal is one which:

(1) Normally requires an environmental impact statement, or

(2) Normally does not require either an environmental impact statement or an environmental assessment (categorical exclusion).

(b) If the proposed action is not covered by paragraph (a) of this section, prepare an environmental assessment (§ 1508.9). The agency shall involve environmental agencies, applicants, and the public, to the extent practicable, in preparing assessments required by § 1508.9(a)(1).

(c) Based on the environmental assessment make its determination whether to prepare an environmental impact statement.

(d) Commence the scoping process (§ 1501.7), if the agency will prepare an environmental impact statement.

(e) Prepare a finding of no significant impact (§ 1508.13), if the agency determines on the basis of the environmental assessment not to prepare a statement.

(1) The agency shall make the finding of no significant impact available

to the affected public as specified in § 1506.6.

(2) In certain limited circumstances, which the agency may cover in its procedures under § 1507.3, the agency shall make the finding of no significant impact available for public review (including State and areawide clearinghouses) for 30 days before the agency makes its final determination whether to prepare an environmental impact statement and before the action may begin. The circumstances are:

(i) The proposed action is, or is closely similar to, one which normally requires the preparation of an environmental impact statement under the procedures adopted by the agency pursuant to § 1507.3, or

(ii) The nature of the proposed action is one without precedent.

### § 1501.5 Lead agencies.

(a) A lead agency shall supervise the preparation of an environmental impact statement if more than one Federal agency either:

(1) Proposes or is involved in the same action; or

(2) Is involved in a group of actions directly related to each other because of their functional interdependence or geographical proximity.

(b) Federal, State, or local agencies, including at least one Federal agency, may act as joint lead agencies to prepare an environmental impact statement (§ 1506.2).

(c) If an action falls within the provisions of paragraph (a) of this section the potential lead agencies shall determine by letter or memorandum which agency shall be the lead agency and which shall be cooperating agencies. The agencies shall resolve the lead agency question so as not to cause delay. If there is disagreement among the agencies, the following factors (which are listed in order of descending importance) shall determine lead agency designation:

(1) Magnitude of agency's involvement.

(2) Project approval/disapproval authority.

(3) Expertise concerning the action's environmental effects.

(4) Duration of agency's involvement.

(5) Sequence of agency's involvement.

(d) Any Federal agency, or any State or local agency or private person substantially affected by the absence of lead agency designation, may make a written request to the potential lead agencies that a lead agency be designated.

(e) If Federal agencies are unable to agree on which agency will be the lead agency or if the procedure described in paragraph (c) of this section has not resulted within 45 days in a lead agency designation, any of the agencies or persons concerned may file a request with the Council asking it to determine which Federal agency shall be the lead agency.

A copy of the request shall be transmitted to each potential lead agency. The request shall consist of:

(1) A precise description of the nature and extent of the proposed action.

(2) A detailed statement of why each potential lead agency should or should not be the lead agency under the criteria specified in paragraph (c) of this section.

(f) A response may be filed by any potential lead agency concerned within 20 days after a request is filed with the Council. The Council shall determine as soon as possible but not later than 20 days after receiving the request and all responses to it which Federal agency shall be the lead agency and which other Federal agencies shall be cooperating agencies.

[43 FR 55992, Nov. 29, 1978; 44 FR 873, Jan. 3, 1979]

### § 1501.6 Cooperating agencies.

The purpose of this section is to emphasize agency cooperation early in the NEPA process. Upon request of the lead agency, any other Federal agency which has jurisdiction by law shall be a cooperating agency. In addition any other Federal agency which has special expertise with respect to any environmental issue, which should be addressed in the statement may be a cooperating agency upon request of the lead agency. An agency may re-uest the lead agency to designate it a ooperating agency.

(a) The lead agency shall:

(1) Request the participation of each ooperating agency in the NEPA proc- s at the earliest possible time.

(2) Use the environmental analysis nd proposals of cooperating agencies ith jurisdiction by law or special ex- ertise, to the maximum extent possi- e consistent with its responsibility as ad agency.

(3) Meet with a cooperating agency t the latter's request.

(b) Each cooperating agency shall:

(1) Participate in the NEPA process t the earliest possible time.

(2) Participate in the scoping process lescribed below in § 1501.7).

(3) Assume on request of the lead gency responsibility for developing formation and preparing environ- ental analyses including portions of e environmental impact statement oncerning which the cooperating gency has special expertise.

(4) Make available staff support at e lead agency's request to enhance e latter's interdisciplinary capabil- y.

(5) Normally use its own funds. The ad agency shall, to the extent avail- ble funds permit, fund those major ctivities or analyses it requests from ooperating agencies. Potential lead gencies shall include such funding re- uirements in their budget requests.

(c) A cooperating agency may in re- ponse to a lead agency's request for sistance in preparing the environ- ental impact statement (described in aragraph (b)(3), (4), or (5) of this sec- on) reply that other program com- itments preclude any involvement or e degree of involvement requested in e action that is the subject of the vironmental impact statement. A opy of this reply shall be submitted o the Council.

### 1501.7 Scoping.

There shall be an early and open rocess for determining the scope of sues to be addressed and for identify- g the significant issues related to a roposed action. This process shall be ermed scoping. As soon as practicable fter its decision to prepare an envi- onmental impact statement and before the scoping process the lead agency shall publish a notice of intent (§ 1508.22) in the FEDERAL REGISTER except as provided in § 1507.3(e).

(a) As part of the scoping process the lead agency shall:

(1) Invite the participation of affected Federal, State, and local agencies, any affected Indian tribe, the proponent of the action, and other interested persons (including those who might not be in accord with the action on environmental grounds), unless there is a limited exception under § 1507.3(c). An agency may give notice in accordance with § 1506.6.

(2) Determine the scope (§ 1508.25) and the significant issues to be analyzed in depth in the environmental impact statement.

(3) Identify and eliminate from detailed study the issues which are not significant or which have been covered by prior environmental review (§ 1506.3), narrowing the discussion of these issues in the statement to a brief presentation of why they will not have a significant effect on the human environment or providing a reference to their coverage elsewhere.

(4) Allocate assignments for preparation of the environmental impact statement among the lead and cooperating agencies, with the lead agency retaining responsibility for the statement.

(5) Indicate any public environmental assessments and other environmental impact statements which are being or will be prepared that are related to but are not part of the scope of the impact statement under consideration.

(6) Identify other environmental review and consultation requirements so the lead and cooperating agencies may prepare other required analyses and studies concurrently with, and integrated with, the environmental impact statement as provided in § 1502.25.

(7) Indicate the relationship between the timing of the preparation of environmental analyses and the agency's tentative planning and decisionmaking schedule.

(b) As part of the scoping process the lead agency may:

(1) Set page limits on environmental documents (§ 1502.7).

(2) Set time limits (§ 1501.8).

(3) Adopt procedures under § 1507.3 to combine its environmental assessment process with its scoping process.

(4) Hold an early scoping meeting or meetings which may be integrated with any other early planning meeting the agency has. Such a scoping meeting will often be appropriate when the impacts of a particular action are confined to specific sites.

(c) An agency shall revise the determinations made under paragraphs (a) and (b) of this section if substantial changes are made later in the proposed action, or if significant new circumstances or information arise which bear on the proposal or its impacts.

### § 1501.8 Time limits.

Although the Council has decided that prescribed universal time limits for the entire NEPA process are too inflexible, Federal agencies are encouraged to set time limits appropriate to individual actions (consistent with the time intervals required by § 1506.10). When multiple agencies are involved the reference to agency below means lead agency.

(a) The agency shall set time limits if an applicant for the proposed action requests them: *Provided,* That the limits are consistent with the purposes of NEPA and other essential considerations of national policy.

(b) The agency may:

(1) Consider the following factors in determining time limits:

(i) Potential for environmental harm.

(ii) Size of the proposed action.

(iii) State of the art of analytic techniques.

(iv) Degree of public need for the proposed action, including the consequences of delay.

(v) Number of persons and agencies affected.

(vi) Degree to which relevant information is known and if not known the time required for obtaining it.

(vii) Degree to which the action is controversial.

(viii) Other time limits imposed on the agency by law, regulations, or executive order.

(2) Set overall time limits or limits for each constituent part of the NEPA process, which may include:

(i) Decision on whether to prepare an environmental impact statement (if not already decided).

(ii) Determination of the scope of the environmental impact statement.

(iii) Preparation of the draft environmental impact statement.

(iv) Review of any comments on the draft environmental impact statement from the public and agencies.

(v) Preparation of the final environmental impact statement.

(vi) Review of any comments on the final environmental impact statement.

(vii) Decision on the action based in part on the environmental impact statement.

(3) Designate a person (such as the project manager or a person in the agency's office with NEPA responsibilities) to expedite the NEPA process.

(c) State or local agencies or members of the public may request a Federal Agency to set time limits.

## PART 1502—ENVIRONMENTAL IMPACT STATEMENT

Sec.

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1502.23 Cost-benefit analysis.
1502.24 Methodology and scientific accuracy.
1502.25 Environmental review and consultation requirements.

AUTHORITY: NEPA, the Environmental Quality Improvement Act of 1970, as amended (42 U.S.C. 4371 *et seq.*), sec. 309 of the Clean Air Act, as amended (42 U.S.C. 7609), and E.O. 11514 (Mar. 5, 1970, as amended by E.O. 11991, May 24, 1977).

SOURCE: 43 FR 55994, Nov. 29, 1978, unless otherwise noted.

### § 1502.1 Purpose.

The primary purpose of an environmental impact statement is to serve as an action-forcing device to insure that the policies and goals defined in the Act are infused into the ongoing programs and actions of the Federal Government. It shall provide full and fair discussion of significant environmental impacts and shall inform decisionmakers and the public of the reasonable alternatives which would avoid or minimize adverse impacts or enhance the quality of the human environment. Agencies shall focus on significant environmental issues and alternatives and shall reduce paperwork and the accumulation of extraneous background data. Statements shall be concise, clear, and to the point, and shall be supported by evidence that the agency has made the necessary environmental analyses. An environmental impact statement is more than a disclosure document. It shall be used by Federal officials in conjunction with other relevant material to plan actions and make decisions.

### § 1502.2 Implementation.

To achieve the purposes set forth in § 1502.1 agencies shall prepare environmental impact statements in the following manner:

(a) Environmental impact statements shall be analytic rather than encyclopedic.

(b) Impacts shall be discussed in proportion to their significance. There shall be only brief discussion of other than significant issues. As in a finding of no significant impact, there should be only enough discussion to show why more study is not warranted.

(c) Environmental impact statements shall be kept concise and shall be no longer than absolutely necessary to comply with NEPA and with these regulations. Length should vary first with potential environmental problems and then with project size.

(d) Environmental impact statements shall state how alternatives considered in it and decisions based on it will or will not achieve the requirements of sections 101 and 102(1) of the Act and other environmental laws and policies.

(e) The range of alternatives discussed in environmental impact statements shall encompass those to be considered by the ultimate agency decisionmaker.

(f) Agencies shall not commit resources prejudicing selection of alternatives before making a final decision (§ 1506.1).

(g) Environmental impact statements shall serve as the means of assessing the environmental impact of proposed agency actions, rather than justifying decisions already made.

### § 1502.3 Statutory requirements for statements.

As required by sec. 102(2)(C) of NEPA environmental impact statements (§ 1508.11) are to be included in every recommendation or report.
On proposals (§ 1508.23).
For legislation and (§ 1508.17).
Other major Federal actions (§ 1508.18).
Significantly (§ 1508.27).
Affecting (§§ 1508.3, 1508.8).
The quality of the human environment (§ 1508.14).

### § 1502.4 Major Federal actions requiring the preparation of environmental impact statements.

(a) Agencies shall make sure the proposal which is the subject of an environmental impact statement is properly defined. Agencies shall use the criteria for scope (§ 1508.25) to determine which proposal(s) shall be the subject of a particular statement. Proposals or parts of proposals which are related to each other closely enough to be, in effect, a single course of action shall be evaluated in a single impact statement.

(b) Environmental impact statements may be prepared, and are sometimes required, for broad Federal actions such as the adoption of new agency programs or regulations (§ 1508.18). Agencies shall prepare statements on broad actions so that they are relevant to policy and are timed to coincide with meaningful points in agency planning and decisionmaking.

(c) When preparing statements on broad actions (including proposals by more than one agency), agencies may find it useful to evaluate the proposal(s) in one of the following ways:

(1) Geographically, including actions occurring in the same general location, such as body of water, region, or metropolitan area.

(2) Generically, including actions which have relevant similarities, such as common timing, impacts, alternatives, methods of implementation, media, or subject matter.

(3) By stage of technological development including federal or federally assisted research, development or demonstration programs for new technologies which, if applied, could significantly affect the quality of the human environment. Statements shall be prepared on such programs and shall be available before the program has reached a stage of investment or commitment to implementation likely to determine subsequent development or restrict later alternatives.

(d) Agencies shall as appropriate employ scoping (§ 1501.7), tiering (§ 1502.20), and other methods listed in §§ 1500.4 and 1500.5 to relate broad and narrow actions and to avoid duplication and delay.

### § 1502.5 Timing.

An agency shall commence preparation of an environmental impact statement as close as possible to the time the agency is developing or is presented with a proposal (§ 1508.23) so that preparation can be completed in time for the final statement to be included in any recommendation or report on the proposal. The statement shall be prepared early enough so that it can serve practically as an important contribution to the decisionmaking process and will not be used to rationalize or justify decisions already made (§§ 1500.2(c), 1501.2, and 1502.2). For instance:

(a) For projects directly undertaken by Federal agencies the environmental impact statement shall be prepared at the feasibility analysis (go-no go) stage and may be supplemented at a later stage if necessary.

(b) For applications to the agency appropriate environmental assessments or statements shall be commenced no later than immediately after the application is received. Federal agencies are encouraged to begin preparation of such assessments or statements earlier, preferably jointly with applicable State or local agencies.

(c) For adjudication, the final environmental impact statement shall normally precede the final staff recommendation and that portion of the public hearing related to the impact study. In appropriate circumstances the statement may follow preliminary hearings designed to gather information for use in the statements.

(d) For informal rulemaking the draft environmental impact statement shall normally accompany the proposed rule.

### § 1502.6 Interdisciplinary preparation.

Environmental impact statements shall be prepared using an inter-disciplinary approach which will insure the integrated use of the natural and social sciences and the environmental design arts (section 102(2)(A) of the Act). The disciplines of the preparers shall be appropriate to the scope and issues identified in the scoping process (§ 1501.7).

### § 1502.7 Page limits.

The text of final environmental impact statements (e.g., paragraphs (d) through (g) of § 1502.10) shall normally be less than 150 pages and for proposals of unusual scope or complexity shall normally be less than 300 pages.

### § 1502.8 Writing.

Environmental impact statements shall be written in plain language and may use appropriate graphics so that decisionmakers and the public can readily understand them. Agencies should employ writers of clear prose or editors to write, review, or edit statements, which will be based upon the analysis and supporting data from the natural and social sciences and the environmental design arts.

### § 1502.9 Draft, final, and supplemental statements.

Except for proposals for legislation as provided in § 1506.8 environmental impact statements shall be prepared in two stages and may be supplemented.

(a) Draft environmental impact statements shall be prepared in accordance with the scope decided upon in the scoping process. The lead agency shall work with the cooperating agencies and shall obtain comments as required in Part 1503 of this chapter. The draft statement must fulfill and satisfy to the fullest extent possible the requirements established for final statements in section 102(2)(C) of the Act. If a draft statement is so inadequate as to preclude meaningful analysis, the agency shall prepare and circulate a revised draft of the appropriate portion. The agency shall make every effort to disclose and discuss at appropriate points in the draft statement all major points of view on the environmental impacts of the alternatives including the proposed action.

(b) Final environmental impact statements shall respond to comments as required in Part 1503 of this chapter. The agency shall discuss at appropriate points in the final statement any responsible opposing view which was not adequately discussed in the draft statement and shall indicate the agency's response to the issues raised.

(c) Agencies:

(1) Shall prepare supplements to either draft or final environmental impact statements if:

(i) The agency makes substantial changes in the proposed action that are relevant to environmental concerns; or

(ii) There are significant new circumstances or information relevant to environmental concerns and bearing on the proposed action or its impacts.

(2) May also prepare supplements when the agency determines that the purposes of the Act will be furthered by doing so.

(3) Shall adopt procedures for introducing a supplement into its formal administrative record, if such a record exists.

(4) Shall prepare, circulate, and file a supplement to a statement in the same fashion (exclusive of scoping) as a draft and final statement unless alternative procedures are approved by the Council.

### § 1502.10 Recommended format.

Agencies shall use a format for environmental impact statements which will encourage good analysis and clear presentation of the alternatives including the proposed action. The following standard format for environmental impact statements should be followed unless the agency determines that there is a compelling reason to do otherwise:

(a) Cover sheet.

(b) Summary.

(c) Table of contents.

(d) Purpose of and need for action.

(e) Alternatives including proposed action (sections 102(2)(C)(iii) and 102(2)(E) of the Act).

(f) Affected environment.

(g) Environmental consequences (especially sections 102(2)(C)(i), (ii), (iv), and (v) of the Act).

(h) List of preparers.

(i) List of Agencies, Organizations, and persons to whom copies of the statement are sent.

(j) Index.

(k) Appendices (if any).

If a different format is used, it shall include paragraphs (a), (b), (c), (h), (i), and (j), of this section and shall include the substance of paragraphs (d), (e), (f), (g), and (k) of this section, as further described in §§ 1502.11 through 1502.18, in any appropriate format.

### § 1502.11 Cover sheet.

The cover sheet shall not exceed one page. It shall include:

(a) A list of the responsible agencies including the lead agency and any cooperating agencies.

(b) The title of the proposed action that is the subject of the statement (and if appropriate the titles of related cooperating agency actions), together with the State(s) and county(ies) (or other jurisdiction if applicable) where the action is located.

(c) The name, address, and telephone number of the person at the agency who can supply further information.

(d) A designation of the statement as a draft, final, or draft or final supplement.

(e) A one paragraph abstract of the statement.

(f) The date by which comments must be received (computed in cooperation with EPA under § 1506.10).

The information required by this section may be entered on Standard Form 424 (in items 4, 6, 7, 10, and 18).

### § 1502.12 Summary.

Each environmental impact statement shall contain a summary which adequately and accurately summarizes the statement. The summary shall stress the major conclusions, areas of controversy (including issues raised by agencies and the public), and the issues to be resolved (including the choice among alternatives). The summary will normally not exceed 15 pages.

### § 1502.13 Purpose and need.

The statement shall briefly specify the underlying purpose and need to which the agency is responding in proposing the alternatives including the proposed action.

### § 1502.14 Alternatives including the proposed action.

This section is the heart of the environmental impact statement. Based on the information and analysis presented in the sections on the Affected Environment (§ 1502.15) and the Environmental Consequences (§ 1502.16), it should present the environmental impacts of the proposal and the alternatives in comparative form, thus sharply defining the issues and providing a clear basis for choice among options by the decisionmaker and the public. In this section agencies shall:

(a) Rigorously explore and objectively evaluate all reasonable alternatives, and for alternatives which were eliminated from detailed study, briefly discuss the reasons for their having been eliminated.

(b) Devote substantial treatment to each alternative considered in detail including the proposed action so that reviewers may evaluate their comparative merits.

(c) Include reasonable alternatives not within the jurisdiction of the lead agency.

(d) Include the alternative of no action.

(e) Identify the agency's preferred alternative or alternatives, if one or more exists, in the draft statement and identify such alternative in the final statement unless another law prohibits the expression of such a preference.

(f) Include appropriate mitigation measures not already included in the proposed action or alternatives.

### § 1502.15 Affected environment.

The environmental impact statement shall succinctly describe the environment of the area(s) to be affected or created by the alternatives under consideration. The descriptions shall be no longer than is necessary to understand the effects of the alternatives. Data and analyses in a statement shall be commensurate with the importance of the impact, with less important material summarized, consolidated, or simply referenced. Agencies shall avoid useless bulk in statements and shall concentrate effort and attention on important issues. Verbose descriptions of the affected environment are themselves no measure of the adequacy of an environmental impact statement.

### § 1502.16 Environmental consequences.

This section forms the scientific and analytic basis for the comparisons under § 1502.14. It shall consolidate the discussions of those elements required by sections 102(2)(C)(i), (ii), (iv), and (v) of NEPA which are within the scope of the statement and as much of section 102(2)(C)(iii) as is necessary to support the comparisons. The discussion will include the environmental impacts of the alternatives including the proposed action, any adverse environmental effects which cannot be avoided should the proposal be implemented, the relationship between short-term uses of man's environment and the maintenance and enhancement of long-term productivity, and any irreversible or irretrievable commitments of resources which would be involved in the proposal should it be implemented. This section should not duplicate discussions in § 1502.14. It shall include discussions of:

(a) Direct effects and their significance (§ 1508.8).

(b) Indirect effects and their significance (§ 1508.8).

(c) Possible conflicts between the proposed action and the objectives of Federal, regional, State, and local (and in the case of a reservation, Indian tribe) land use plans, policies and controls for the area concerned. (See § 1506.2(d).)

(d) The environmental effects of alternatives including the proposed action. The comparisons under § 1502.14 will be based on this discussion.

(e) Energy requirements and conservation potential of various alternatives and mitigation measures.

(f) Natural or depletable resource requirements and conservation potential of various alternatives and mitigation measures.

(g) Urban quality, historic and cultural resources, and the design of the built environment, including the reuse and conservation potential of various alternatives and mitigation measures.

(h) Means to mitigate adverse environmental impacts (if not fully covered under § 1502.14(f)).

[43 FR 55994, Nov. 29, 1978; 44 FR 873, Jan. 3, 1979]

### § 1502.17 List of preparers.

The environmental impact statement shall list the names, together with their qualifications (expertise, experience, professional disciplines), of the persons who were primarily responsible for preparing the environmental impact statement or significant background papers, including basic components of the statement (§§ 1502.6 and 1502.8). Where possible the persons who are responsible for a particular analysis, including analyses in background papers, shall be identified. Normally the list will not exceed two pages.

### § 1502.18 Appendix.

If an agency prepares an appendix to an environmental impact statement the appendix shall:

(a) Consist of material prepared in connection with an environmental impact statement (as distinct from material which is not so prepared and which is incorporated by reference (§ 1502.21)).

(b) Normally consist of material which substantiates any analysis fundamental to the impact statement.

(c) Normally be analytic and relevant to the decision to be made.

(d) Be circulated with the environmental impact statement or be readily available on request.

### § 1502.19 Circulation of the environmental impact statement.

Agencies shall circulate the entire draft and final environmental impact statements except for certain appendices as provided in § 1502.18(d) and unchanged statements as provided in § 1503.4(c). However, if the statement is unusually long, the agency may circulate the summary instead, except that the entire statement shall be furnished to:

(a) Any Federal agency which has jurisdiction by law or special expertise with respect to any environmental impact involved and any appropriate Federal, State or local agency authorized to develop and enforce environmental standards.

(b) The applicant, if any.

(c) Any person, organization, or agency requesting the entire environmental impact statement.

(d) In the case of a final environmental impact statement any person, organization, or agency which submitted substantive comments on the draft.

If the agency circulates the summary and thereafter receives a timely request for the entire statement and for additional time to comment, the time for that requestor only shall be extended by at least 15 days beyond the minimum period.

### § 1502.20 Tiering.

Agencies are encouraged to tier their environmental impact statements to eliminate repetitive discussions of the same issues and to focus on the actual issues ripe for decision at each level of environmental review (§ 1508.28). Whenever a broad environmental impact statement has been prepared (such as a program or policy statement) and a subsequent statement or environmental assessment is then prepared on an action included within the entire program or policy (such as a site specific action) the subsequent statement or environmental assessment need only summarize the issues discussed in the broader statement and incorporate discussions from the broader statement by reference and shall concentrate on the issues specific to the subsequent action. The subsequent document shall state where the earlier document is available. Tiering may also be appropriate for different stages of actions. (Section 1508.28).

### § 1502.21 Incorporation by reference.

Agencies shall incorporate material into an environmental impact statement by reference when the effect will be to cut down on bulk without impeding agency and public review of the action. The incorporated material shall be cited in the statement and its content briefly described. No material may be incorporated by reference unless it is reasonably available for inspection by potentially interested persons within the time allowed for comment. Material based on proprietary data which is itself not available for review and comment shall not be incorporated by reference.

### § 1502.22 Incomplete or unavailable information.

When an agency is evaluating reasonably foreseeable significant adverse effects on the human environment in an environmental impact statement and there is incomplete or unavailable information, the agency shall always make clear that such information is lacking.

(a) If the incomplete information relevant to reasonably foreseeable significant adverse impacts is essential to a reasoned choice among alternatives and the overall costs of obtaining it are not exorbitant, the agency shall include the information in the environmental impact statement.

(b) If the information relevant to reasonably foreseeable significant adverse impacts cannot be obtained because the overall costs of obtaining it are exorbitant or the means to obtain it are not known, the agency shall include within the environmental impact statement:

(1) A statement that such information is incomplete or unavailable; (2) a statement of the relevance of the incomplete or unavailable information to evaluating reasonably foreseeable significant adverse impacts on the human environment; (3) a summary of existing credible scientific evidence which is relevant to evaluating the reasonably foreseeable significant adverse impacts on the human environment, and (4) the agency's evaluation of such impacts based upon theoretical approaches or research methods generally accepted in the scientific community. For the purposes of this section, "reasonably foreseeable" includes impacts which have catastrophic consequences, even if their probability of occurrence is low, provided that the analysis of the impacts is supported by credible scientific evidence, is not based on pure conjecture, and is within the rule of reason.

(c) The amended regulation will be applicable to all environmental impact statements for which a Notice of Intent (40 CFR 1508.22) is published in the FEDERAL REGISTER on or after May 27, 1986. For environmental impact statements in progress, agencies may choose to comply with the requirements of either the original or amended regulation.

[51 FR 15625, Apr. 25, 1986]

### § 1502.23 Cost-benefit analysis.

If a cost-benefit analysis relevant to the choice among environmentally different alternatives is being considered for the proposed action, it shall be incorporated by reference or appended to the statement as an aid in evaluating the environmental consequences. To assess the adequacy of compliance with section 102(2)(B) of the Act the statement shall, when a cost-benefit analysis is prepared, discuss the relationship between that analysis and any analyses of unquantified environmental impacts, values, and amenities. For purposes of complying with the Act, the weighing of the merits and drawbacks of the various alternatives need not be displayed in a monetary cost-benefit analysis and should not be when there are important qualitative considerations. In any event, an environmental impact statement should at least indicate those considerations, including factors not related to environmental quality, which are likely to be relevant and important to a decision.

### § 1502.24 Methodology and scientific accuracy.

Agencies shall insure the professional integrity, including scientific integrity, of the discussions and analyses in environmental impact statements. They shall identify any methodologies used and shall make explicit reference by footnote to the scientific and other sources relied upon for conclusions in the statement. An agency may place discussion of methodology in an appendix.

### § 1502.25 Environmental review and consultation requirements.

(a) To the fullest extent possible, agencies shall prepare draft environmental impact statements concurrently with and integrated with environmental impact analyses and related surveys and studies required by the Fish and Wildlife Coordination Act (16 U.S.C. 661 *et seq.*), the National Historic Preservation Act of 1966 (16 U.S.C. 470 *et seq.*), the Endangered Species Act of 1973 (16 U.S.C. 1531 et seq.), and other environmental review laws and executive orders.

(b) The draft environmental impact statement shall list all Federal permits, licenses, and other entitlements which must be obtained in implementing the proposal. If it is uncertain whether a Federal permit, license, or other entitlement is necessary, the draft environmental impact statement shall so indicate.

## PART 1503—COMMENTING

Sec.
1503.1 Inviting comments.
1503.2 Duty to comment.
1503.3 Specificity of comments.
1503.4 Response to comments.

AUTHORITY: NEPA, the Environmental Quality Improvement Act of 1970, as amended (42 U.S.C. 4371 *et seq.*), sec. 309 of the Clean Air Act, as amended (42 U.S.C. 7609), and E.O. 11514 (Mar. 5, 1970, as amended by E.O. 11991, May 24, 1977).

SOURCE: 43 FR 55997, Nov. 29, 1978, unless otherwise noted.

### § 1503.1 Inviting comments.

(a) After preparing a draft environmental impact statement and before preparing a final environmental impact statement the agency shall:

(1) Obtain the comments of any Federal agency which has jurisdiction by law or special expertise with respect to any environmental impact involved or which is authorized to develop and enforce environmental standards.

(2) Request the comments of:

(i) Appropriate State and local agencies which are authorized to develop and enforce environmental standards;

(ii) Indian tribes, when the effects may be on a reservation; and

(iii) Any agency which has requested that it receive statements on actions of the kind proposed.

Office of Management and Budget Circular A-95 (Revised), through its system of clearinghouses, provides a means of securing the views of State and local environmental agencies. The clearinghouses may be used, by mutual agreement of the lead agency and the clearinghouse, for securing

State and local reviews of the draft environmental impact statements.

(3) Request comments from the applicant, if any.

(4) Request comments from the public, affirmatively soliciting comments from those persons or organizations who may be interested or affected.

(b) An agency may request comments on a final environmental impact statement before the decision is finally made. In any case other agencies or persons may make comments before the final decision unless a different time is provided under § 1506.10.

### § 1503.2 Duty to comment.

Federal agencies with jurisdiction by law or special expertise with respect to any environmental impact involved and agencies which are authorized to develop and enforce environmental standards shall comment on statements within their jurisdiction, expertise, or authority. Agencies shall comment within the time period specified for comment in § 1506.10. A Federal agency may reply that it has no comment. If a cooperating agency is satisfied that its views are adequately reflected in the environmental impact statement, it should reply that it has no comment.

### § 1503.3 Specificity of comments.

(a) Comments on an environmental impact statement or on a proposed action shall be as specific as possible and may address either the adequacy of the statement or the merits of the alternatives discussed or both.

(b) When a commenting agency criticizes a lead agency's predictive methodology, the commenting agency should describe the alternative methodology which it prefers and why.

(c) A cooperating agency shall specify in its comments whether it needs additional information to fulfill other applicable environmental reviews or consultation requirements and what information it needs. In particular, it shall specify any additional information it needs to comment adequately on the draft statement's analysis of significant site-specific effects associated with the granting or approving by that cooperating agency of necessary Federal permits, licenses, or entitlements.

(d) When a cooperating agency with jurisdiction by law objects to or expresses reservations about the proposal on grounds of environmental impacts, the agency expressing the objection or reservation shall specify the mitigation measures it considers necessary to allow the agency to grant or approve applicable permit, license, or related requirements or concurrences.

### § 1503.4 Response to comments.

(a) An agency preparing a final environmental impact statement shall assess and consider comments both individually and collectively, and shall respond by one or more of the means listed below, stating its response in the final statement. Possible responses are to:

(1) Modify alternatives including the proposed action.

(2) Develop and evaluate alternatives not previously given serious consideration by the agency.

(3) Supplement, improve, or modify its analyses.

(4) Make factual corrections.

(5) Explain why the comments do not warrant further agency response, citing the sources, authorities, or reasons which support the agency's position and, if appropriate, indicate those circumstances which would trigger agency reappraisal or further response.

(b) All substantive comments received on the draft statement (or summaries thereof where the response has been exceptionally voluminous), should be attached to the final statement whether or not the comment is thought to merit individual discussion by the agency in the text of the statement.

(c) If changes in response to comments are minor and are confined to the responses described in paragraphs (a)(4) and (5) of this section, agencies may write them on errata sheets and attach them to the statement instead of rewriting the draft statement. In such cases only the comments, the responses, and the changes and not the final statement need be circulated (§ 1502.19). The entire document with a new cover sheet shall be filed as the final statement (§ 1506.9).

## PART 1504—PREDECISION REFERRALS TO THE COUNCIL OF PROPOSED FEDERAL ACTIONS DETERMINED TO BE ENVIRONMENTALLY UNSATISFACTORY

Sec.
1504.1 Purpose.
1504.2 Criteria for referral.
1504.3 Procedure for referrals and response.

AUTHORITY: NEPA, the Environmental Quality Improvement Act of 1970, as amended (42 U.S.C. 4371 *et seq.*), sec. 309 of the Clean Air Act, as amended (42 U.S.C. 7609), and E.O. 11514 (Mar. 5, 1970, as amended by E.O. 11991, May 24, 1977).

SOURCE: 43 FR 55998, Nov. 29, 1978, unless otherwise noted.

### § 1504.1 Purpose.

(a) This part establishes procedures for referring to the Council Federal interagency disagreements concerning proposed major Federal actions that might cause unsatisfactory environmental effects. It provides means for early resolution of such disagreements.

(b) Under section 309 of the Clean Air Act (42 U.S.C. 7609), the Administrator of the Environmental Protection Agency is directed to review and comment publicly on the environmental impacts of Federal activities, including actions for which environmental impact statements are prepared. If after this review the Administrator determines that the matter is "unsatisfactory from the standpoint of public health or welfare or environmental quality," section 309 directs that the matter be referred to the Council (hereafter "environmental referrals").

(c) Under section 102(2)(C) of the Act other Federal agencies may make similar reviews of environmental impact statements, including judgments on the acceptability of anticipated environmental impacts. These reviews must be made available to the President, the Council and the public.

### § 1504.2 Criteria for referral.

Environmental referrals should be made to the Council only after concerted, timely (as early as possible in the process), but unsuccessful attempts to resolve differences with the lead agency. In determining what environmental objections to the matter are appropriate to refer to the Council, an agency should weigh potential adverse environmental impacts, considering:

(a) Possible violation of national environmental standards or policies.

(b) Severity.

(c) Geographical scope.

(d) Duration.

(e) Importance as precedents.

(f) Availability of environmentally preferable alternatives.

### § 1504.3 Procedure for referrals and response.

(a) A Federal agency making the referral to the Council shall:

(1) Advise the lead agency at the earliest possible time that it intends to refer a matter to the Council unless a satisfactory agreement is reached.

(2) Include such advice in the referring agency's comments on the draft environmental impact statement, except when the statement does not contain adequate information to permit an assessment of the matter's environmental acceptability.

(3) Identify any essential information that is lacking and request that it be made available at the earliest possible time.

(4) Send copies of such advice to the Council.

(b) The referring agency shall deliver its referral to the Council not later than twenty-five (25) days after the final environmental impact statement has been made available to the Environmental Protection Agency, commenting agencies, and the public. Except when an extension of this period has been granted by the lead agency, the Council will not accept a referral after that date.

(c) The referral shall consist of:

(1) A copy of the letter signed by the head of the referring agency and delivered to the lead agency informing the lead agency of the referral and the reasons for it, and requesting that no action be taken to implement the matter until the Council acts upon the

referral. The letter shall include a copy of the statement referred to in (c)(2) of this section.

(2) A statement supported by factual evidence leading to the conclusion that the matter is unsatisfactory from the standpoint of public health or welfare or environmental quality. The statement shall:

(i) Identify any material facts in controversy and incorporate (by reference if appropriate) agreed upon facts,

(ii) Identify any existing environmental requirements or policies which would be violated by the matter,

(iii) Present the reasons why the referring agency believes the matter is environmentally unsatisfactory,

(iv) Contain a finding by the agency whether the issue raised is of national importance because of the threat to national environmental resources or policies or for some other reason,

(v) Review the steps taken by the referring agency to bring its concerns to the attention of the lead agency at the earliest possible time, and

(vi) Give the referring agency's recommendations as to what mitigation alternative, further study, or other course of action (including abandonment of the matter) are necessary to remedy the situation.

(d) Not later than twenty-five (25) days after the referral to the Council the lead agency may deliver a response to the Council, and the referring agency. If the lead agency requests more time and gives assurance that the matter will not go forward in the interim, the Council may grant an extension. The response shall:

(1) Address fully the issues raised in the referral.

(2) Be supported by evidence.

(3) Give the lead agency's response to the referring agency's recommendations.

(e) Interested persons (including the applicant) may deliver their views in writing to the Council. Views in support of the referral should be delivered not later than the referral. Views in support of the response shall be delivered not later than the response.

(f) Not later than twenty-five (25) days after receipt of both the referral and any response or upon being informed that there will be no response (unless the lead agency agrees to a longer time), the Council may take one or more of the following actions:

(1) Conclude that the process of referral and response has successfully resolved the problem.

(2) Initiate discussions with the agencies with the objective of mediation with referring and lead agencies.

(3) Hold public meetings or hearings to obtain additional views and information.

(4) Determine that the issue is not one of national importance and request the referring and lead agencies to pursue their decision process.

(5) Determine that the issue should be further negotiated by the referring and lead agencies and is not appropriate for Council consideration until one or more heads of agencies report to the Council that the agencies' disagreements are irreconcilable.

(6) Publish its findings and recommendations (including where appropriate a finding that the submitted evidence does not support the position of an agency).

(7) When appropriate, submit the referral and the response together with the Council's recommendation to the President for action.

(g) The Council shall take no longer than 60 days to complete the actions specified in paragraph (f)(2), (3), or (5) of this section.

(h) When the referral involves an action required by statute to be determined on the record after opportunity for agency hearing, the referral shall be conducted in a manner consistent with 5 U.S.C. 557(d) (Administrative Procedure Act).

[43 FR 55998, Nov. 29, 1978; 44 FR 873, Jan. 3, 1979]

## PART 1505—NEPA AND AGENCY DECISIONMAKING

Sec.
1505.1 Agency decisionmaking procedures.
1505.2 Record of decision in cases requiring environmental impact statements.
1505.3 Implementing the decision.

AUTHORITY: NEPA, the Environmental Quality Improvement Act of 1970, as amended (42 U.S.C. 4371 *et seq.*), sec. 309 of the Clean Air Act, as amended (42 U.S.C. 7609), and E.O. 11514 (Mar. 5, 1970, as amended by E.O. 11991, May 24, 1977).

SOURCE: 43 FR 55990, Nov. 29, 1978, unless otherwise noted.

### § 1505.1 Agency decisionmaking procedures.

Agencies shall adopt procedures (§ 1507.3) to ensure that decisions are made in accordance with the policies and purposes of the Act. Such procedures shall include but not be limited to:

(a) Implementing procedures under section 102(2) to achieve the requirements of sections 101 and 102(1).

(b) Designating the major decision points for the agency's principal programs likely to have a significant effect on the human environment and assuring that the NEPA process corresponds with them.

(c) Requiring that relevant environmental documents, comments, and responses be part of the record in formal rulemaking or adjudicatory proceedings.

(d) Requiring that relevant environmental documents, comments, and responses accompany the proposal through existing agency review processes so that agency officials use the statement in making decisions.

(e) Requiring that the alternatives considered by the decisionmaker are encompassed by the range of alternatives discussed in the relevant environmental documents and that the decisionmaker consider the alternatives described in the environmental impact statement. If another decision document accompanies the relevant environmental documents to the decisionmaker, agencies are encouraged to make available to the public before the decision is made any part of that document that relates to the comparison of alternatives.

### § 1505.2 Record of decision in cases requiring environmental impact statements.

At the time of its decision (§ 1506.10) or, if appropriate, its recommendation to Congress, each agency shall prepare a concise public record of decision. The record, which may be integrated into any other record prepared by the agency, including that required by OMB Circular A-95 (Revised), part I, sections 6(c) and (d), and Part II, section 5(b)(4), shall:

(a) State what the decision was.

(b) Identify all alternatives considered by the agency in reaching its decision, specifying the alternative or alternatives which were considered to be environmentally preferable. An agency may discuss preferences among alternatives based on relevant factors including economic and technical considerations and agency statutory missions. An agency shall identify and discuss all such factors including any essential considerations of national policy which were balanced by the agency in making its decision and state how those considerations entered into its decision.

(c) State whether all practicable means to avoid or minimize environmental harm from the alternative selected have been adopted, and if not, why they were not. A monitoring and enforcement program shall be adopted and summarized where applicable for any mitigation.

### § 1505.3 Implementing the decision.

Agencies may provide for monitoring to assure that their decisions are carried out and should do so in important cases. Mitigation (§ 1505.2(c)) and other conditions established in the environmental impact statement or during its review and committed as part of the decision shall be implemented by the lead agency or other appropriate consenting agency. The lead agency shall:

(a) Include appropriate conditions in grants, permits or other approvals.

(b) Condition funding of actions on mitigation.

(c) Upon request, inform cooperating or commenting agencies on progress in carrying out mitigation measures which they have proposed and which were adopted by the agency making the decision.

(d) Upon request, make available to the public the results of relevant monitoring.

# PART 1506—OTHER REQUIREMENTS OF NEPA

Sec.
1506.1 Limitations on actions during NEPA process.
1506.2 Elimination of duplication with State and local procedures.
1506.3 Adoption.
1506.4 Combining documents.
1506.5 Agency responsibility.
1506.6 Public involvement.
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1506.8 Proposals for legislation.
1506.9 Filing requirements.
1506.10 Timing of agency action.
1506.11 Emergencies.
1506.12 Effective date.

AUTHORITY: NEPA, the Environmental Quality Improvement Act of 1970, as amended (42 U.S.C. 4371 *et seq.*), sec. 309 of the Clean Air Act, as amended (42 U.S.C. 7609), and E.O. 11514 (Mar. 5, 1970, as amended by E.O. 11991, May 24, 1977).

SOURCE: 43 FR 56000, Nov. 29, 1978, unless otherwise noted.

## § 1506.1 Limitations on actions during NEPA process.

(a) Until an agency issues a record of decision as provided in § 1505.2 (except as provided in paragraph (c) of this section), no action concerning the proposal shall be taken which would:

(1) Have an adverse environmental impact; or

(2) Limit the choice of reasonable alternatives.

(b) If any agency is considering an application from a non-Federal entity, and is aware that the applicant is about to take an action within the agency's jurisdiction that would meet either of the criteria in paragraph (a) of this section, then the agency shall promptly notify the applicant that the agency will take appropriate action to insure that the objectives and procedures of NEPA are achieved.

(c) While work on a required program environmental impact statement is in progress and the action is not covered by an existing program statement, agencies shall not undertake in the interim any major Federal action covered by the program which may significantly affect the quality of the human environment unless such action:

(1) Is justified independently of the program;

(2) Is itself accompanied by an adequate environmental impact statement; and

(3) Will not prejudice the ultimate decision on the program. Interim action prejudices the ultimate decision on the program when it tends to determine subsequent development or limit alternatives.

(d) This section does not preclude development by applicants of plans or designs or performance of other work necessary to support an application for Federal, State or local permits or assistance. Nothing in this section shall preclude Rural Electrification Administration approval of minimal expenditures not affecting the environment (*e.g.* long leadtime equipment and purchase options) made by nongovernmental entities seeking loan guarantees from the Administration.

## § 1506.2 Elimination of duplication with State and local procedures.

(a) Agencies authorized by law to cooperate with State agencies of statewide jurisdiction pursuant to section 102(2)(D) of the Act may do so.

(b) Agencies shall cooperate with State and local agencies to the fullest extent possible to reduce duplication between NEPA and State and local requirements, unless the agencies are specifically barred from doing so by some other law. Except for cases covered by paragraph (a) of this section, such cooperation shall to the fullest extent possible include:

(1) Joint planning processes.

(2) Joint environmental research and studies.

(3) Joint public hearings (except where otherwise provided by statute).

(4) Joint environmental assessments.

(c) Agencies shall cooperate with State and local agencies to the fullest extent possible to reduce duplication between NEPA and comparable State and local requirements, unless the agencies are specifically barred from doing so by some other law. Except for cases covered by paragraph (a) of this section, such cooperation shall to the fullest extent possible include joint environmental impact statements. In such cases one or more Federal agencies and one or more State or local agencies shall be joint lead agencies. Where State laws or local ordinances have environmental impact statement requirements in addition to but not in conflict with those in NEPA, Federal agencies shall cooperate in fulfilling these requirements as well as those of Federal laws so that one document will comply with all applicable laws.

(d) To better integrate environmental impact statements into State or local planning processes, statements shall discuss any inconsistency of a proposed action with any approved State or local plan and laws (whether or not federally sanctioned). Where an inconsistency exists, the statement should describe the extent to which the agency would reconcile its proposed action with the plan or law.

### § 1506.3 Adoption.

(a) An agency may adopt a Federal draft or final environmental impact statement or portion thereof provided that the statement or portion thereof meets the standards for an adequate statement under these regulations.

(b) If the actions covered by the original environmental impact statement and the proposed action are substantially the same, the agency adopting another agency's statement is not required to recirculate it except as a final statement. Otherwise the adopting agency shall treat the statement as a draft and recirculate it (except as provided in paragraph (c) of this section).

(c) A cooperating agency may adopt without recirculating the environmental impact statement of a lead agency when, after an independent review of the statement, the cooperating agency concludes that its comments and suggestions have been satisfied.

(d) When an agency adopts a statement which is not final within the agency that prepared it, or when the action it assesses is the subject of a referral under Part 1504, or when the statement's adequacy is the subject of a judicial action which is not final, the agency shall so specify.

### § 1506.4 Combining documents.

Any environmental document in compliance with NEPA may be combined with any other agency document to reduce duplication and paperwork.

### § 1506.5 Agency responsibility.

(a) *Information.* If an agency requires an applicant to submit environmental information for possible use by the agency in preparing an environmental impact statement, then the agency should assist the applicant by outlining the types of information required. The agency shall independently evaluate the information submitted and shall be responsible for its accuracy. If the agency chooses to use the information submitted by the applicant in the environmental impact statement, either directly or by reference, then the names of the persons responsible for the independent evaluation shall be included in the list of preparers (§ 1502.17). It is the intent of this paragraph that acceptable work not be redone, but that it be verified by the agency.

(b) *Environmental assessments.* If an agency permits an applicant to prepare an environmental assessment, the agency, besides fulfilling the requirements of paragraph (a) of this section, shall make its own evaluation of the environmental issues and take responsibility for the scope and content of the environmental assessment.

(c) *Environmental impact statements.* Except as provided in §§ 1506.2 and 1506.3 any environmental impact statement prepared pursuant to the requirements of NEPA shall be prepared directly by or by a contractor selected by the lead agency or where appropriate under § 1501.6(b), a cooperating agency. It is the intent of these regulations that the contractor be chosen solely by the lead agency, or by the lead agency in cooperation with cooperating agencies, or where appropriate by a cooperating agency to avoid any conflict of interest. Contractors shall execute a disclosure statement prepared by the lead agency, or where appropriate the cooperating agency, specifying that they have no financial or other interest in the outcome of the project. If the document is prepared by contract, the responsible Federal official shall furnish guidance and participate in the preparation and shall independently evaluate the statement prior to its approval and take responsibility for its scope and contents. Nothing in this section is intended to prohibit any agency from requesting any person to submit information to it or to prohibit any person from submitting information to any agency.

### § 1506.6 Public involvement.

Agencies shall:

(a) Make diligent efforts to involve the public in preparing and implementing their NEPA procedures.

(b) Provide public notice of NEPA-related hearings, public meetings, and the availability of environmental documents so as to inform those persons and agencies who may be interested or affected.

(1) In all cases the agency shall mail notice to those who have requested it on an individual action.

(2) In the case of an action with effects of national concern notice shall include publication in the FEDERAL REGISTER and notice by mail to national organizations reasonably expected to be interested in the matter and may include listing in the *102 Monitor.* An agency engaged in rulemaking may provide notice by mail to national organizations who have requested that notice regularly be provided. Agencies shall maintain a list of such organizations.

(3) In the case of an action with effects primarily of local concern the notice may include:

(i) Notice to State and areawide clearinghouses pursuant to OMB Circular A-95 (Revised).

(ii) Notice to Indian tribes when effects may occur on reservations.

(iii) Following the affected State's public notice procedures for comparable actions.

(iv) Publication in local newspapers (in papers of general circulation rather than legal papers).

(v) Notice through other local media.

(vi) Notice to potentially interested community organizations including small business associations.

(vii) Publication in newsletters that may be expected to reach potentially interested persons.

(viii) Direct mailing to owners and occupants of nearby or affected property.

(ix) Posting of notice on and off site in the area where the action is to be located.

(c) Hold or sponsor public hearings or public meetings whenever appropriate or in accordance with statutory requirements applicable to the agency. Criteria shall include whether there is:

(1) Substantial environmental controversy concerning the proposed action or substantial interest in holding the hearing.

(2) A request for a hearing by another agency with jurisdiction over the action supported by reasons why a hearing will be helpful. If a draft environmental impact statement is to be considered at a public hearing, the agency should make the statement available to the public at least 15 days in advance (unless the purpose of the hearing is to provide information for the draft environmental impact statement).

(d) Solicit appropriate information from the public.

(e) Explain in its procedures where interested persons can get information or status reports on environmental impact statements and other elements of the NEPA process.

(f) Make environmental impact statements, the comments received, and any underlying documents available to the public pursuant to the provisions of the Freedom of Information Act (5 U.S.C. 552), without regard to the exclusion for interagency memoranda where such memoranda transmit comments of Federal agencies on the environmental impact of the proposed action. Materials to be made available to the public shall be provided to the public without charge to the extent practicable, or at a fee which is not more than the actual costs of reproducing copies required to be sent to other Federal agencies, including the Council.

### § 1506.7 Further guidance.

The Council may provide further guidance concerning NEPA and its procedures including:

(a) A handbook which the Council may supplement from time to time, which shall in plain language provide guidance and instructions concerning the application of NEPA and these regulations.

(b) Publication of the Council's Memoranda to Heads of Agencies.

(c) In conjunction with the Environmental Protection Agency and the publication of the 102 Monitor, notice of:

(1) Research activities;

(2) Meetings and conferences related to NEPA; and

(3) Successful and innovative procedures used by agencies to implement NEPA.

### § 1506.8 Proposals for legislation.

(a) The NEPA process for proposals for legislation (§ 1508.17) significantly affecting the quality of the human environment shall be integrated with the legislative process of the Congress. A legislative environmental impact statement is the detailed statement required by law to be included in a recommendation or report on a legislative proposal to Congress. A legislative environmental impact statement shall be considered part of the formal transmittal of a legislative proposal to Congress; however, it may be transmitted to Congress up to 30 days later in order to allow time for completion of an accurate statement which can serve as the basis for public and Congressional debate. The statement must be available in time for Congressional hearings and deliberations.

(b) Preparation of a legislative environmental impact statement shall conform to the requirements of these regulations except as follows:

(1) There need not be a scoping process.

(2) The legislative statement shall be prepared in the same manner as a draft statement, but shall be considered the "detailed statement" required by statute; *Provided*, That when any of the following conditions exist both the draft and final environmental impact statement on the legislative proposal shall be prepared and circulated as provided by §§ 1503.1 and 1506.10.

(i) A Congressional Committee with jurisdiction over the proposal has a rule requiring both draft and final environmental impact statements.

(ii) The proposal results from a study process required by statute (such as those required by the Wild and Scenic Rivers Act (16 U.S.C. 1271 *et seq.*) and the Wilderness Act (16 U.S.C. 1131 *et seq.*)).

(iii) Legislative approval is sought for Federal or federally assisted construction or other projects which the agency recommends be located at specific geographic locations. For proposals requiring an environmental impact statement for the acquisition of space by the General Services Administration, a draft statement shall accompany the Prospectus or the 11(b) Report of Building Project Surveys to the Congress, and a final statement shall be completed before site acquisition.

(iv) The agency decides to prepare draft and final statements.

(c) Comments on the legislative statement shall be given to the lead agency which shall forward them along with its own responses to the Congressional committees with jurisdiction.

### § 1506.9 Filing requirements.

Environmental impact statements together with comments and responses shall be filed with the Environmental Protection Agency, attention Office of Federal Activities (A-104), 401 M Street SW., Washington, DC 20460. Statements shall be filed with EPA no earlier than they are also transmitted to commenting agencies and made available to the public. EPA shall deliver one copy of each statement to the Council, which shall satisfy the requirement of availability to the President. EPA may issue guidelines to agencies to implement its responsibilities under this section and § 1506.10.

### § 1506.10 Timing of agency action.

(a) The Environmental Protection Agency shall publish a notice in the FEDERAL REGISTER each week of the environmental impact statements filed during the preceding week. The minimum time periods set forth in this section shall be calculated from the date of publication of this notice.

(b) No decision on the proposed action shall be made or recorded under § 1505.2 by a Federal agency until the later of the following dates:

(1) Ninety (90) days after publication of the notice described above in paragraph (a) of this section for a draft environmental impact statement.

(2) Thirty (30) days after publication of the notice described above in paragraph (a) of this section for a final environmental impact statement.

An exception to the rules on timing may be made in the case of an agency decision which is subject to a formal internal appeal. Some agencies have a formally established appeal process which allows other agencies or the public to take appeals on a decision and make their views known, after publication of the final environmental impact statement. In such cases, where a real opportunity exists to alter the decision, the decision may be made and recorded at the same time the environmental impact statement is published. This means that the period for appeal of the decision and the 30-day period prescribed in paragraph (b)(2) of this section may run concurrently. In such cases the environmental impact statement shall explain the timing and the public's right of appeal. An agency engaged in rulemaking under the Administrative Procedure Act or other statute for the purpose of protecting the public health or safety, may waive the time period in paragraph (b)(2) of this section and publish a decision on the final rule simultaneously with publication of the notice of the availability of the final environmental impact statement as described in paragraph (a) of this section.

(c) If the final environmental impact statement is filed within ninety (90) days after a draft environmental impact statement is filed with the Environmental Protection Agency, the minimum thirty (30) day period and the minimum ninety (90) day period may run concurrently. However, subject to paragraph (d) of this section agencies shall allow not less than 45 days for comments on draft statements.

(d) The lead agency may extend prescribed periods. The Environmental Protection Agency may upon a showing by the lead agency of compelling reasons of national policy reduce the prescribed periods and may upon a showing by any other Federal agency of compelling reasons of national policy also extend prescribed periods, but only after consultation with the lead agency. (Also see § 1507.3(d).) Failure to file timely comments shall not be a sufficient reason for extending a period. If the lead agency does not concur with the extension of time, EPA may not extend it for more than 30 days. When the Environmental Protection Agency reduces or extends any period of time it shall notify the Council.

[43 FR 56000, Nov. 29, 1978; 44 FR 874, Jan. 3, 1979]

### § 1506.11 Emergencies.

Where emergency circumstances make it necessary to take an action with significant environmental impact without observing the provisions of these regulations, the Federal agency taking the action should consult with the Council about alternative arrangements. Agencies and the Council will limit such arrangements to actions necessary to control the immediate impacts of the emergency. Other actions remain subject to NEPA review.

### § 1506.12 Effective date.

The effective date of these regulations is July 30, 1979, except that for agencies that administer programs that qualify under section 102(2)(D) of the Act or under section 104(h) of the Housing and Community Development Act of 1974 an additional four months shall be allowed for the State or local agencies to adopt their implementing procedures.

(a) These regulations shall apply to the fullest extent practicable to ongoing activities and environmental documents begun before the effective date. These regulations do not apply to an environmental impact statement or supplement if the draft statement was filed before the effective date of these regulations. No completed environmental documents need be redone by reasons of these regulations. Until these regulations are applicable, the Council's guidelines published in the FEDERAL REGISTER of August 1, 1973, shall continue to be applicable. In cases where these regulations are applicable the guidelines are superseded. However, nothing shall prevent an agency from proceeding under these regulations at an earlier time.

(b) NEPA shall continue to be applicable to actions begun before January 1, 1970, to the fullest extent possible.

## PART 1507—AGENCY COMPLIANCE

Sec.

1507.1 Compliance.
1507.2 Agency capability to comply.
1507.3 Agency procedures.

AUTHORITY: NEPA, the Environmental Quality Improvement Act of 1970, as amended (42 U.S.C. 4371 *et seq.*), sec. 309 of the Clean Air Act, as amended (42 U.S.C. 7609), and E.O. 11514 (Mar. 5, 1970, as amended by E.O. 11991, May 24, 1977).

SOURCE: 43 FR 56002, Nov. 29, 1978, unless otherwise noted.

### § 1507.1 Compliance.

All agencies of the Federal Government shall comply with these regulations. It is the intent of these regulations to allow each agency flexibility in adapting its implementing procedures authorized by § 1507.3 to the requirements of other applicable laws.

### § 1507.2 Agency capability to comply.

Each agency shall be capable (in terms of personnel and other resources) of complying with the requirements enumerated below. Such compliance may include use of other's resources, but the using agency shall itself have sufficient capability to evaluate what others do for it. Agencies shall:

(a) Fulfill the requirements of section 102(2)(A) of the Act to utilize a systematic, interdisciplinary approach which will insure the integrated use of the natural and social sciences and the environmental design arts in planning and in decisionmaking which may have an impact on the human environment. Agencies shall designate a person to be responsible for overall review of agency NEPA compliance.

(b) Identify methods and procedures required by section 102(2)(B) to insure that presently unquantified environmental amenities and values may be given appropriate consideration.

(c) Prepare adequate environmental impact statements pursuant to section 102(2)(C) and comment on statements in the areas where the agency has jurisdiction by law or special expertise or is authorized to develop and enforce environmental standards.

(d) Study, develop, and describe alternatives to recommended courses of action in any proposal which involves unresolved conflicts concerning alternative uses of available resources. This requirement of section 102(2)(E) extends to all such proposals, not just the more limited scope of section 102(2)(C)(iii) where the discussion of alternatives is confined to impact statements.

(e) Comply with the requirements of section 102(2)(H) that the agency initiate and utilize ecological information in the planning and development of resource-oriented projects.

(f) Fulfill the requirements of sections 102(2)(F), 102(2)(G), and 102(2)(I), of the Act and of Executive Order 11514, Protection and Enhancement of Environmental Quality, Sec. 2.

### § 1507.3 Agency procedures.

(a) Not later than eight months after publication of these regulations as finally adopted in the FEDERAL REGISTER, or five months after the establishment of an agency, whichever shall come later, each agency shall as necessary adopt procedures to supplement these regulations. When the agency is a department, major subunits are encouraged (with the consent of the department) to adopt their own procedures. Such procedures shall not paraphrase these regulations. They shall confine themselves to implementing procedures. Each agency shall consult with the Council while developing its procedures and before publishing them in the FEDERAL REGISTER for comment. Agencies with similar programs should consult with each other and the Council to coordinate their procedures, especially for programs requesting similar information from applicants. The procedures shall be adopted only after an opportunity for public review and after review by the Council for conformity with the Act and these regulations. The Council shall complete its review within 30 days. Once in effect they shall be filed with the Council and made readily available to the public. Agencies are encouraged to publish explanatory guidance for these regulations and their own procedures. Agencies shall continue to review their policies and procedures and in consultation with the Council to revise them as necessary to ensure full compliance with the purposes and provisions of the Act.

(b) Agency procedures shall comply with these regulations except where compliance would be inconsistent with statutory requirements and shall include:

(1) Those procedures required by §§ 1501.2(d), 1502.9(c)(3), 1505.1, 1506.6(e), and 1508.4.

(2) Specific criteria for and identification of those typical classes of action:

(i) Which normally do require environmental impact statements.

(ii) Which normally do not require either an environmental impact statement or an environmental assessment (categorical exclusions (§ 1508.4)).

(iii) Which normally require environmental assessments but not necessarily environmental impact statements.

(c) Agency procedures may include specific criteria for providing limited exceptions to the provisions of these regulations for classified proposals. They are proposed actions which are specifically authorized under criteria established by an Executive Order or statute to be kept secret in the interest of national defense or foreign policy and are in fact properly classified pursuant to such Executive Order or statute. Environmental assessments and environmental impact statements which address classified proposals may be safeguarded and restricted from public dissemination in accordance with agencies' own regulations applicable to classified information. These documents may be organized so that classified portions can be included as annexes, in order that the unclassified portions can be made available to the public.

(d) Agency procedures may provide for periods of time other than those presented in § 1506.10 when necessary to comply with other specific statutory requirements.

(e) Agency procedures may provide that where there is a lengthy period between the agency's decision to prepare an environmental impact statement and the time of actual preparation, the notice of intent required by § 1501.7 may be published at a reasonable time in advance of preparation of the draft statement.

## PART 1508—TERMINOLOGY AND INDEX

Sec.

1508.1 Terminology.
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AUTHORITY: NEPA, the Environmental Quality Improvement Act of 1970, as amended (42 U.S.C. 4371 *et seq.*), sec. 309 of the Clean Air Act, as amended (42 U.S.C. 7609), and E.O. 11514 (Mar. 5, 1970, as amended by E.O. 11991, May 24, 1977).

SOURCE: 43 FR 56003, Nov. 29, 1978, unless otherwise noted.

### § 1508.1 Terminology.

The terminology of this part shall be uniform throughout the Federal Government.

### § 1508.2 Act.

"Act" means the National Environmental Policy Act, as amended (42 U.S.C. 4321, *et seq.*) which is also referred to as "NEPA."

### § 1508.3 Affecting.

"Affecting" means will or may have an effect on.

### § 1508.4 Categorical exclusion.

"Categorical exclusion" means a category of actions which do not individually or cumulatively have a significant effect on the human environment and which have been found to have no such effect in procedures adopted by a Federal agency in implementation of these regulations (§ 1507.3) and for which, therefore, neither an environmental assessment nor an environmental impact statement is required. An agency may decide in its procedures or otherwise, to prepare environmental assessments for the reasons stated in § 1508.9 even though it is not required to do so. Any procedures under this section shall provide for extraordinary circumstances in which a normally excluded action may have a significant environmental effect.

### § 1508.5 Cooperating agency.

"Cooperating agency" means any Federal agency other than a lead agency which has jurisdiction by law or special expertise with respect to any environmental impact involved in a proposal (or a reasonable alternative) for legislation or other major Federal action significantly affecting the quality of the human environment. The selection and responsibilities of a cooperating agency are described in § 1501.6. A State or local agency of similar qualifications or, when the effects are on a reservation, an Indian Tribe, may by agreement with the lead agency become a cooperating agency.

### § 1508.6 Council.

"Council" means the Council on Environmental Quality established by Title II of the Act.

### § 1508.7 Cumulative impact.

"Cumulative impact" is the impact on the environment which results from the incremental impact of the action when added to other past, present, and reasonably foreseeable future actions regardless of what agency (Federal or non-Federal) or person undertakes such other actions. Cumulative impacts can result from individually minor but collectively significant actions taking place over a period of time.

### § 1508.8 Effects.

"Effects" include:

(a) Direct effects, which are caused by the action and occur at the same time and place.

(b) Indirect effects, which are caused by the action and are later in time or farther removed in distance, but are still reasonably foreseeable. Indirect effects may include growth inducing effects and other effects related to induced changes in the pattern of land use, population density or growth rate, and related effects on air and water and other natural systems, including ecosystems.

Effects and impacts as used in these regulations are synonymous. Effects includes ecological (such as the effects on natural resources and on the components, structures, and functioning of affected ecosystems), aesthetic, historic, cultural, economic, social, or health, whether direct, indirect, or cumulative. Effects may also include those resulting from actions which may have both beneficial and detrimental effects, even if on balance the agency believes that the effect will be beneficial.

### § 1508.9 Environmental assessment.

"Environmental assessment":

(a) Means a concise public document for which a Federal agency is responsible that serves to:

(1) Briefly provide sufficient evidence and analysis for determining

whether to prepare an environmental impact statement or a finding of no significant impact.

(2) Aid an agency's compliance with the Act when no environmental impact statement is necessary.

(3) Facilitate preparation of a statement when one is necessary.

(b) Shall include brief discussions of the need for the proposal, of alternatives as required by section 102(2)(E), of the environmental impacts of the proposed action and alternatives, and a listing of agencies and persons consulted.

### § 1508.10 Environmental document.

"Environmental document" includes the documents specified in § 1508.9 (environmental assessment), § 1508.11 (environmental impact statement), § 1508.13 (finding of no significant impact), and § 1508.22 (notice of intent).

### § 1508.11 Environmental impact statement.

"Environmental impact statement" means a detailed written statement as required by section 102(2)(C) of the Act.

### § 1508.12 Federal agency.

"Federal agency" means all agencies of the Federal Government. It does not mean the Congress, the Judiciary, or the President, including the performance of staff functions for the President in his Executive Office. It also includes for purposes of these regulations States and units of general local government and Indian tribes assuming NEPA responsibilities under section 104(h) of the Housing and Community Development Act of 1974.

### § 1508.13 Finding of no significant impact.

"Finding of no significant impact" means a document by a Federal agency briefly presenting the reasons why an action, not otherwise excluded (§ 1508.4), will not have a significant effect on the human environment and for which an environmental impact statement therefore will not be prepared. It shall include the environmental assessment or a summary of it and shall note any other environmental documents related to it (§ 1501.7(a)(5)). If the assessment is included, the finding need not repeat any of the discussion in the assessment but may incorporate it by reference.

### § 1508.14 Human environment.

"Human environment" shall be interpreted comprehensively to include the natural and physical environment and the relationship of people with that environment. (See the definition of "effects" (§ 1508.8).) This means that economic or social effects are not intended by themselves to require preparation of an environmental impact statement. When an environmental impact statement is prepared and economic or social and natural or physical environmental effects are interrelated, then the environmental impact statement will discuss all of these effects on the human environment.

### § 1508.15 Jurisdiction by law.

"Jurisdiction by law" means agency authority to approve, veto, or finance all or part of the proposal.

### § 1508.16 Lead agency.

"Lead agency" means the agency or agencies preparing or having taken primary responsibility for preparing the environmental impact statement.

### § 1508.17 Legislation.

"Legislation" includes a bill or legislative proposal to Congress developed by or with the significant cooperation and support of a Federal agency, but does not include requests for appropriations. The test for significant cooperation is whether the proposal is in fact predominantly that of the agency rather than another source. Drafting does not by itself constitute significant cooperation. Proposals for legislation include requests for ratification of treaties. Only the agency which has primary responsibility for the subject matter involved will prepare a legislative environmental impact statement.

### § 1508.18 Major Federal action.

"Major Federal action" includes actions with effects that may be major and which are potentially subject to

Federal control and responsibility. Major reinforces but does not have a meaning independent of significantly (§ 1508.27). Actions include the circumstance where the responsible officials fail to act and that failure to act is reviewable by courts or administrative tribunals under the Administrative Procedure Act or other applicable law as agency action.

(a) Actions include new and continuing activities, including projects and programs entirely or partly financed, assisted, conducted, regulated, or approved by federal agencies; new or revised agency rules, regulations, plans, policies, or procedures; and legislative proposals (§§ 1506.8, 1508.17). Actions do not include funding assistance solely in the form of general revenue sharing funds, distributed under the State and Local Fiscal Assistance Act of 1972, 31 U.S.C. 1221 *et seq.*, with no Federal agency control over the subsequent use of such funds. Actions do not include bringing judicial or administrative civil or criminal enforcement actions.

(b) Federal actions tend to fall within one of the following categories:

(1) Adoption of official policy, such as rules, regulations, and interpretations adopted pursuant to the Administrative Procedure Act, 5 U.S.C. 551 *et seq.*; treaties and international conventions or agreements; formal documents establishing an agency's policies which will result in or substantially alter agency programs.

(2) Adoption of formal plans, such as official documents prepared or approved by federal agencies which guide or prescribe alternative uses of Federal resources, upon which future agency actions will be based.

(3) Adoption of programs, such as a group of concerted actions to implement a specific policy or plan; systematic and connected agency decisions allocating agency resources to implement a specific statutory program or executive directive.

(4) Approval of specific projects, such as construction or management activities located in a defined geographic area. Projects include actions approved by permit or other regulatory decision as well as federal and federally assisted activities.

### § 1508.19 Matter.

"Matter" includes for purposes of Part 1504:

(a) With respect to the Environmental Protection Agency, any proposed legislation, project, action or regulation as those terms are used in section 309(a) of the Clean Air Act (42 U.S.C. 7609).

(b) With respect to all other agencies, any proposed major federal action to which section 102(2)(C) of NEPA applies.

### § 1508.20 Mitigation.

"Mitigation" includes:

(a) Avoiding the impact altogether by not taking a certain action or parts of an action.

(b) Minimizing impacts by limiting the degree or magnitude of the action and its implementation.

(c) Rectifying the impact by repairing, rehabilitating, or restoring the affected environment.

(d) Reducing or eliminating the impact over time by preservation and maintenance operations during the life of the action.

(e) Compensating for the impact by replacing or providing substitute resources or environments.

### § 1508.21 NEPA process.

"NEPA process" means all measures necessary for compliance with the requirements of section 2 and Title I of NEPA.

### § 1508.22 Notice of intent.

"Notice of intent" means a notice that an environmental impact statement will be prepared and considered. The notice shall briefly:

(a) Describe the proposed action and possible alternatives.

(b) Describe the agency's proposed scoping process including whether, when, and where any scoping meeting will be held.

(c) State the name and address of a person within the agency who can answer questions about the proposed action and the environmental impact statement.

### § 1508.23 Proposal.

"Proposal" exists at that stage in the development of an action when an agency subject to the Act has a goal and is actively preparing to make a decision on one or more alternative means of accomplishing that goal and the effects can be meaningfully evaluated. Preparation of an environmental impact statement on a proposal should be timed (§ 1502.5) so that the final statement may be completed in time for the statement to be included in any recommendation or report on the proposal. A proposal may exist in fact as well as by agency declaration that one exists.

### § 1508.24 Referring agency.

"Referring agency" means the federal agency which has referred any matter to the Council after a determination that the matter is unsatisfactory from the standpoint of public health or welfare or environmental quality.

### § 1508.25 Scope.

Scope consists of the range of actions, alternatives, and impacts to be considered in an environmental impact statement. The scope of an individual statement may depend on its relationships to other statements (§§1502.20 and 1508.28). To determine the scope of environmental impact statements, agencies shall consider 3 types of actions, 3 types of alternatives, and 3 types of impacts. They include:

(a) Actions (other than unconnected single actions) which may be:

(1) Connected actions, which means that they are closely related and therefore should be discussed in the same impact statement. Actions are connected if they:

(i) Automatically trigger other actions which may require environmental impact statements.

(ii) Cannot or will not proceed unless other actions are taken previously or simultaneously.

(iii) Are interdependent parts of a larger action and depend on the larger action for their justification.

(2) Cumulative actions, which when viewed with other proposed actions have cumulatively significant impacts and should therefore be discussed in the same impact statement.

(3) Similar actions, which when viewed with other reasonably foreseeable or proposed agency actions, have similarities that provide a basis for evaluating their environmental consequencies together, such as common timing or geography. An agency may wish to analyze these actions in the same impact statement. It should do so when the best way to assess adequately the combined impacts of similar actions or reasonable alternatives to such actions is to treat them in a single impact statement.

(b) Alternatives, which include: (1) No action alternative.

(2) Other reasonable courses of actions.

(3) Mitigation measures (not in the proposed action).

(c) Impacts, which may be: (1) Direct; (2) indirect; (3) cumulative.

### § 1508.26 Special expertise.

"Special expertise" means statutory responsibility, agency mission, or related program experience.

### § 1508.27 Significantly.

"Significantly" as used in NEPA requires considerations of both context and intensity:

(a) *Context.* This means that the significance of an action must be analyzed in several contexts such as society as a whole (human, national), the affected region, the affected interests, and the locality. Significance varies with the setting of the proposed action. For instance, in the case of a site-specific action, significance would usually depend upon the effects in the locale rather than in the world as a whole. Both short- and long-term effects are relevant.

(b) *Intensity.* This refers to the severity of impact. Responsible officials must bear in mind that more than one agency may make decisions about partial aspects of a major action. The following should be considered in evaluating intensity:

(1) Impacts that may be both beneficial and adverse. A significant effect may exist even if the Federal agency

believes that on balance the effect will be beneficial.

(2) The degree to which the proposed action affects public health or safety.

(3) Unique characteristics of the geographic area such as proximity to historic or cultural resources, park lands, prime farmlands, wetlands, wild and scenic rivers, or ecologically critical areas.

(4) The degree to which the effects on the quality of the human environment are likely to be highly controversial.

(5) The degree to which the possible effects on the human environment are highly uncertain or involve unique or unknown risks.

(6) The degree to which the action may establish a precedent for future actions with significant effects or represents a decision in principle about a future consideration.

(7) Whether the action is related to other actions with individually insignificant but cumulatively significant impacts. Significance exists if it is reasonable to anticipate a cumulatively significant impact on the environment. Significance cannot be avoided by terming an action temporary or by breaking it down into small component parts.

(8) The degree to which the action may adversely affect districts, sites, highways, structures, or objects listed in or eligible for listing in the National Register of Historic Places or may cause loss or destruction of significant scientific, cultural, or historical resources.

(9) The degree to which the action may adversely affect an endangered or threatened species or its habitat that has been determined to be critical under the Endangered Species Act of 1973.

(10) Whether the action threatens a violation of Federal, State, or local law or requirements imposed for the protection of the environment.

[43 FR 56003, Nov. 29, 1978; 44 FR 874, Jan. 3, 1979]

### § 1508.28 Tiering.

"Tiering" refers to the coverage of general matters in broader environmental impact statements (such as national program or policy statements) with subsequent narrower statements or environmental analyses (such as regional or basinwide program statements or ultimately site-specific statements) incorporating by reference the general discussions and concentrating solely on the issues specific to the statement subsequently prepared. Tiering is appropriate when the sequence of statements or analyses is:

(a) From a program, plan, or policy environmental impact statement to a program, plan, or policy statement or analysis of lesser scope or to a site-specific statement or analysis.

(b) From an environmental impact statement on a specific action at an early stage (such as need and site selection) to a supplement (which is preferred) or a subsequent statement or analysis at a later stage (such as environmental mitigation). Tiering in such cases is appropriate when it helps the lead agency to focus on the issues which are ripe for decision and exclude from consideration issues already decided or not yet ripe.

## PART 1515—FREEDOM OF INFORMATION ACT PROCEDURES

PURPOSE

Sec.
1515.1 What are these procedure

ORGANIZATION OF CEQ

1515.2 What is the Council on Environmental Quality (CEQ)?
1515.3 How is CEQ organized?

PROCEDURES FOR REQUESTING RECORDS

1515.5 How to make a Freedom of Information Act request.

AVAILABILITY OF INFORMATION

1515.10 What information is available, and how can it be obtained?

COSTS

1515.15 What fees may be charged, and how should they be paid?

AUTHORITY: 5 U.S.C. 552, as amended by Pub. L. 93-502.

SOURCE: 42 FR 65158, Dec. 30, 1977, unless otherwise noted.

## PURPOSE

### § 1515.1 What are these procedures?

The Freedom of Information Act (5 U.S.C. 552, commonly known as FOIA) is a law which creates a procedure for any person to request official documents and other records from United States Government agencies. The law requires every Federal agency to make available to the public the material requested, unless the material falls under one of the limited exceptions stated in section 552(b)(5) of the Act, and the agency has good reason to refuse the request. These procedures explain how the Council on Environmental Quality—one of several offices in the Executive Office of the President—will carry out the Freedom of Information Act. They are written from the standpoint of a member of the public requesting material from the Council.

## ORGANIZATION OF CEQ

### § 1515.2 What is the Council on Environmental Quality (CEQ)?

(a) The Council on Environmental Quality ("CEQ" or "the Council") was created by the National Environmental Policy Act of 1969, as amended (42 U.S.C. 4321 through 4347). The Council's authority is derived from that Act, the Environmental Quality Improvement Act of 1970, as amended (42 U.S.C. 4371-4374), Reorganization Plan No. 1 of 1977 (July 15, 1977), and Executive Order 11514, Protection and Enhancement of Environmental Quality, March 5, 1970, as amended by Executive Order 11991, May 24, 1977.

(b) The Council's primary responsibilities include the following:

(1) To review and evaluate the programs and activities of the Federal Government to determine how they are contributing to the attainment of the national environmental policy;

(2) To assist Federal agencies and departments in appraising the effectiveness of their existing and proposed facilities, programs, policies, and activities affecting environmental quality;

(3) To develop and recommend to the President policies to improve environmental quality to meet the conservation, social, economic, health, and other requirements and goals of the Nation;

(4) To advise and assist the President in achieving international cooperation for dealing with environmental problems;

(5) To assist in coordinating among Federal agencies and departments those programs which affect, protect, and improve environmental quality, including Federal compliance with the environmental impact statement process, and to seek resolution of significant environmental issues;

(6) To foster research relating to environmental quality and the impacts of new or changing technologies; and

(7) To analyze long and short term environmental problems and trends and assist in preparing an annual Environmental Quality Report to the President and the Congress.

(c) The Council maintains a "Quarterly Index" which lists its current policies and procedures, as required by section 552(a)(2) of the Freedom of Information Act. This index is updated and published in the FEDERAL REGISTER quarterly, starting in 1976. The Quarterly Index—and the specific items listed in the index—are available on request from the Freedom of Information Officer. You may also inspect or copy any of these materials at the Council's office during the hours stated below in § 1515.3(f).

### § 1515.3 How is CEQ organized?

(a) The Council is made up of three members appointed by the President and subject to approval by the Senate. One member is designated as chairman by the President. All three serve in a full-time capacity.

(b) The National Environmental Policy Act and the Environmental Quality Improvement Act give the Council the authority to hire any officers and staff that may be necessary to carry out responsibilities and functions specified in these two Acts. Also, the use of consultants and experts is permitted.

(c) In addition to the three members, the Council has program and legal staff.

(d) The Council has no field or regional offices.

(e) The Council has a public affairs office which is responsible for providing information to the general public, the Congress, and the press. If you are interested in general information about the Council or have questions about the Council's recent activities or policy positions, you should call this office at (202) 633-7005 or write to the "Public Affairs Office" of the Council at the address given in the next paragraph.

NOTE: The CEQ public affairs office can respond fully and promptly to most questions you may have; the Council suggests that the Freedom of Information Act procedures be used when you are seeking a specific document and have had difficulty obtaining it.

(f) The Council is located at 722 Jackson Place NW., Washington, DC 20006. Office hours are 9–5:30, Monday through Friday, except legal holidays. If you wish to meet with any of the staff, please write or phone ahead for an appointment. The main number is 202-633-7027.

## PROCEDURES FOR REQUESTING RECORDS

### § 1515.5 How to make a Freedom of Information Act request.

(a) The Chairman has appointed a Freedom of Information Officer who will be responsible for overseeing the Council's administration of the Freedom of Information Act and for receiving, routing, and overseeing the processing of all Freedom of Information requests. The Chairman has also appointed an Appeals Officer who is responsible for processing any appeals.

(b) *Requesting information from the Council.* (1) When you make a Freedom of Information Act request to the Council, the Freedom of Information Officer shall decide how to respond to—or "make an initial determination on"—your request within 10 working days from the date the Officer receives the request. The Freedom of Information Officer will then provide you with written notification of the determination.

(2) You can make a Freedom of Information Act request by writing a letter which states that you are making a Freedom of Information Act request. Address your letter to:

Freedom of Information Officer, Council on Environmental Quality, Executive Office of the President, 722 Jackson Place NW., Washington, DC 20006.

(3) In your request you should identify the desired record or reasonably describe it. The request should be as specific as possible so that the item can be readily found. You should not make blanket requests, such as requests for "the entire file of" or "all materials relating to" a specified subject.

(4) The Council will make a reasonable effort to assist you in defining the request to eliminate extraneous and unwanted materials and to keep search and copying fees to a minimum. If you have budgetary constraints and anticipate that your request might be costly you may wish to indicate the maximum fee you are prepared to pay for acquiring the information. (See § 1515.15(c) also.)

(5) The 10 day period for making a determination on a request will begin when the records reqested are specified or reasonably identifiable.

(6) Despite its name, the Freedom of Information Act does not require a government agency to create or research information that you would like or that you may think the agency should have. The Act only requres that existing records be made available to the public.

(c) *Council's response to a request.* (1) Upon receipt of any request under the Act, the Freedom of Information Officer shall direct the request to the appropriate staff member at the Council, who will review the request and advise the Freedom of Information Officer as soon as possible.

(2) If it is appropriate to grant the request, the staff member will immediately collect the requested materials in order to accompany, wherever possible, the Freedom of Information Officer's letter notifying you of the decision.

(3) If your request is denied, in part or in full, the letter notifying you of the decision will be signed by the Freedom of Information Officer, and will include the names of any other indi-

viduals who participated in the decision. The letter will include the reasons for any denial and the procedure for filing an appeal.

(d) *Appeals.* (1) If you are not satisfied with the response you have received from the Freedom of Information Officer, you may ask the Council to reconsider the decision. You should explain what material you still wish to receive, and why you believe the Council should disclose this to you. This is called an "appeal." You must make you appeal within 45 days of the date on the letter which denied your request.

(2) You can make an appeal by writing a letter to:

FOIA Appeals Officer, Council on Environmental Quality, Executive Office of the President, 722 Jackson Place NW., Washington, DC 20006.

(3) Your letter should specify the records being requested and ask the Appeals Officer to review the determination made by the Freedom of Information Officer. The letter should explain the basis for the appeal.

(4) The Appeals Officer shall decide the appeal—or "make a final determination"—within 20 working days from the date the Officer receives the appeal. The Appeals Officer (or designee) will send you a letter informing you of the decision as soon as it is made. If the Appeals Officer denies your request, in part or in whole, the letter will also notify you of the provisions for judicial review and the names of any persons who participated in the final determination of the appeal.

(e) *Extending the Council's time to respond.* In unusual circumstances, the time limits for response to your request (paragraphs (b) and (d) of this section) may be extended by the Council for not more than 10 working days. Extensions may be granted by the Freedom of Information Officer in the case of initial requests and by the Appeals Officer in the case of any appeals. The extension period may be split between the initial request and the appeal but may not exceed 10 working days overall. Any extension will be made or confirmed to you in writing and will set forth the reasons for the extension and the date that the final determination is expected. The term "unusual circumstances" means:

> (i) The need to search for and collect the requested records from * * * establishments that are separate from the office processing the request;
>
> (ii) The need to search for, collect, and appropriately examine a voluminous amount of separate and distinct records which are demanded in a single request; or
>
> (iii) The need for consultation, which shall be conducted with all practicable speed, with another agency having a substantial interest in the determination of the request or among two or more components of the agency having substantial subject-matter interest therein.

(5 U.S.C. 552(a)(6)(B))

## AVAILABILITY OF INFORMATION

### § 1515.10 What information is available, and how can it be obtained?

(a) When a request for information has been approved, in whole or in part, you may make an appointment to inspect or copy the materials requested during regular business hours by writing or telephoning the Freedom of Information Officer at the address or phone number given in § 1515.3(f). You may be charged reasonable fees for copying materials, as explained by § 1515.15. The Council on Environmental Quality will permit copying of any available material but will reserve the right to limit the number of copies made with the Council's copying facilities.

(b) In general, all records of the Council are available to the public, as required by the Freedom of Information Act. The Council claims the right, where it is applicable, to withhold material under the provisions specified in the Freedom of Information Act as amended (5 U.S.C. 552(b)).

(c) The legislative history of the establishment of the Council states that the Congress intended the Council to be a confidential advisor to the President on matters of environmental policy. Therefore, members of the public should presume that communications between the Council and the President (and their staffs) are confidential and ordinarily will not be released; they will usually fall, at a minimum, within Exemption 5 of the Act

he Freedom of Information Officer
hall review each request, however, to
etermine whether the record is exclu-
vely factual or may have factual por-
ons which may be reasonably segre-
ated and made available to the re-
uester. Furthermore, on the recom-
endation of the FOIA Officer or Ap-
eals Officer, the Council will consider
he release of an entire record, even if
comes within an exemption or con-
ins policy advice, if its disclosure
ould not impair Executive policy-
aking processes or the Council's par-
cipation in decisionmaking.

### COSTS

### 1515.15 What fees may be charged, and how should they be paid?

(a) Following is the schedule of fees
ou may be charged for the search
nd reproduction of information avail-
ble under the Freedom of Informa-
on Act, 5 U.S.C. 552, as amended.

(1) *Search for records.* Five dollars
er hour when the search is conducted
y a clerical employee. Eight dollars
er hour when the search is conducted
y a professional employee. There will
e no charge for searches of less than
ne hour.

(2) *Duplication of records.* Records
ill be duplicated at a rate of $0.10 per
age for copying of 10 pages or more.
here will be no charge for duplicat-
ng 9 pages or less.

(3) *Other.* When no specific fee has
een established for a service, or the
equest for a service does not fall
nder categories (1) and (2), the Ad-
inistrative Officer is authorized to
stablish an appropriate fee based on
direct costs" as provided in the Free-
om of Information Act. Examples of
ervices covered by this provision in-
lude searches involving computer
ime or special travel, transportation,
r communication costs.

(b) If the Council anticipates that
he fees chargeable under this section
ill amount to more than $25, or the
aximum amount specified in your re-
uest, you shall be promptly notified
f the amount of the anticipated fee
r the closest estimate of the amount.
n such instances you will be advised
f your option to consult with Council
ersonnel in order to reformulate the request in a manner which will reduce the fees, yet still meet your needs. A reformulated request shall be considered a new request, thus beginning a new 10 working day period for processing.

(c) Fees must be paid in full prior to issuance of the requested copies. In the event you owe money for previous request, copies of records will not be provided for any subsequent request until the debt has been paid in full.

(d) Search costs are due and payable even if the record which was requested cannot be located after all reasonable efforts have been made, or if the FOI Officer determines that a record which has been requested is exempt under the Freedom of Information Act as amended and is to be withheld.

(e) Payment shall be in the form either of a personal check or bank draft drawn on a bank in the United States, or a postal monev order. Checks shall be made payable to General Services Administration. You should mail or deliver any payment for services to the Administrative Office, Council on Environmental Quality, 722 Jackson Place NW., Washington, DC 20006.

(f) A receipt for fees paid will be given upon request. Refunds of fees paid for services actually rendered will not be made.

(g) The Council may waive all or part of any fee provided for in this section when the Freedom of Information Officer (or designee) deems it to be in either the Council's interest or in the general public's interest.

## PART 1516—PRIVACY ACT IMPLEMENTATION

Sec.

1516.1 Purpose and scope.
1516.2 Definitions.
1516.3 Procedures for requests pertaining to individual records in a record system.
1516.4 Times, places, and requirements for the identification of the individual making a request.
1516.5 Disclosure of requested information to the individual.
1516.6 Request for correction or amendment to the record.
1516.7 Agency review of request for correction or amendment of the record.

Sec.
1516.8 Appeal of an initial adverse agency determination on correction or amendment of the record.
1516.9 Disclosure of a record to a person other than the individual to whom the record pertains.
1516.10 Fees.

AUTHORITY: 5 U.S.C. 552a; Pub. L. 93-579.

SOURCE: 42 FR 32537, June 27, 1977, unless otherwise noted.

### § 1516.1 Purpose and scope.

The purposes of these regulations are to:

(a) Establish a procedure by which an individual can determine if the Council on Environmental Quality (hereafter known as the Council) maintains a system of records which includes a record pertaining to the individual; and

(b) Establish a procedure by which an individual can gain access to a record pertaining to him or her for the purpose of review, amendment and/or correction.

### § 1516.2 Definitons.

For the purpose of these regulations:

(a) The term "individual" means a citizen of the United States or an alien lawfully admited for permanent residence;

(b) The term "maintain" means maintain, collect, use or disseminate;

(c) The term "record" means any item or collection or grouping of information about an individual that is maintained by the Council (including, but not limited to, his or her employment history, payroll information, and financial transactions), and that contains his or her name, or an identifying number, symbol, or other identifying particular assigned to the individual such as a social security number;

(d) The term "system of records" means a group of any records under the control of the Council from which information is retrieved by the name of the individual or by some identifying number, symbol, or other identifying particular assigned to the individual; and

(e) The term "routine use" means with respect to the disclosure of a record, the use of such record for a purpose which is compatible with the purpose for which it was collected.

### § 1516.3 Procedures for requests pertaining to individual records in a record system.

An individual shall submit a written request to the Administrative Officer of the Council to determine if a system of records named by the individual contains a record pertaining to the individual. The individual shall submit a written request to the Administrative Officer of the Council which states the individual's desire to review his or her record. The Administrative Officer of the Council is available to answer questions regarding these regulations and to provide assistance in locating records in the Council's system of records.

[42 FR 32537, June 27, 1977; 42 FR 35960, July 13, 1977]

### § 1516.4 Times, places, and requirements for the identification of the individual making a request.

An individual making a request to the Administrative Officer of the Council pursuant to § 1516.3 shall present the request at the Council's office, 722 Jackson Place NW., Washington, DC 20006, on any business day between the hours of 9 a.m. and 5 p.m. and should be prepared to identify himself by signature. Requests will also be accepted in writing if mailed to the Council's offices and signed by the requester.

### § 1516.5 Disclosure of requested information to the individual.

Upon verification of identity, the Council shall disclose to the individual the information contained in the record which pertains to that individual.

(a) The individual may be accompanied for this purpose by a person of his choosing.

(b) Upon request of the individual to whom the record pertains, all information in the accounting of disclosures will be made available.

[42 FR 35960, July 13, 1977]

### § 1516.6 Request for correction or amendment to the record.

The individual may submit a request to the Administrative Officer of the Council which states the individual's desire to correct or to amend his or her record. This request must be made in accordance with the procedures of § 1516.4 and shall describe in detail the change which is requested.

[42 FR 32537, June 27, 1977. Redesignated at 42 FR 35960, July 13, 1977]

### § 1516.7 Agency review of request for correction or amendment of the record.

Within ten working days of the receipt of a request to correct or to amend a record, the Administrative Officer of the Council will acknowledge in writing such receipt and promptly either:

(a) Make any correction or amendment of any portion thereof which the individual believes is not accurate, relevant, timely, or complete; or

(b) Inform the individual of his or her refusal to correct or amend the record in accordance with the request, the reason for the refusal, and the procedure established by the Council for the individual to request a review of that refusal.

### § 1516.8 Appeal of an initial adverse agency determination on correction or amendment of the record.

An individual may appeal refusal by the Administrative Officer of the Council to correct or to amend his or her record by submitting a request for a review of such refusal to the General Counsel, Council on Environmental Quality, 722 Jackson Place NW., Washington, DC 20006. The General Counsel shall, not later than thirty working days from the date on which the individual requests such a review, complete such review and make a final determination unless, for good cause shown, the General Counsel extends such thirty day period. If, after his or her review, the General Counsel also refuses to correct or to amend the record in accordance with the request, the individual may file with the Council a concise statement setting forth the reasons for his or her disagreement with the General Counsel's decision and may seek judicial relief under 5 U.S.C. 552a(g)(1)(A).

### § 1516.9 Disclosure of a record to a person other than the individual to whom the record pertains.

The Council will not disclose a record to any individual other than to the individual to whom the record pertains without receiving the prior written consent of the individual to whom the record pertains, unless the disclosure either has been listed as a "routine use" in the Council's notices of its systems of records or falls within the special conditions of disclosure set forth in section 3 of the Privacy Act of 1974.

### § 1516.10 Fees.

If an individual requests copies of his or her record, he or she shall be charged ten cents per page, excluding the cost of any search for the record, in advance of receipt of the pages.

## PART 1517—PUBLIC MEETING PROCEDURES OF THE COUNCIL ON ENVIRONMENTAL QUALITY

Sec.

1517.1 Policy and scope.
1517.2 Definitions.
1517.3 Open meeting requirement.
1517.4 Exceptions.
1517.5 Procedure for closing meetings.
1517.6 Notice of meetings.
1517.7 Records of closed meetings.

Authority: 5 U.S.C. 552b(g); Pub. L. 94-409.

Source: 42 FR 20818, Apr. 22, 1977, unless otherwise noted.

### § 1517.1 Policy and scope.

Consistent with the policy that the public is entitled to the fullest information regarding the decisionmaking processes of the Federal Government, it is the purpose of this part to open the meetings of the Council on Environmental Quality to public observation while protecting the rights of individuals and the ability of the Council to carry out its primary responsibility of providing advice to the President. Actions taken by the Chairman acting as Director of the Office of Environmental Quality and Council actions involving advice to the President when such advice is not formulated collegially during a meeting are outside the scope of this part. In addition to conducting the meetings required by this part, it is the Council's policy to conduct, open to public observation, periodic meetings involving Council discussions of Council business, including where appropriate, matters outside the scope of this part. This part does not affect the procedures set forth in Part 1515 pursuant to which records of the Council are made available to the public for inspection and copying, except that the exemptions set forth in § 1517.4(a) shall govern in the case of any request made to copy or inspect the transcripts, recording or minutes described in § 1517.7.

[47 FR 6277, Feb. 11, 1982]

### § 1517.2 Definitions.

For the purpose of this part:

(a) The term "Council" shall mean the Council on Environmental Quality established under Title II of the National Environmental Policy Act of 1969 (42 U.S.C. 4321 through 4347).

(b) The term "meeting" means the deliberations of at least two Council members where such deliberations determine or result in the joint conduct or disposition of official collegial Council business, but does not include deliberations to take actions to open or close a meeting under §§ 1517.4 and 1517.5 or to release or withhold information under §§ 1517.4 and 1517.7. "Meeting" shall not be construed to prevent Council members from considering individually Council business that is circulated to them sequentially in writing.

(c) "Director" means the Chairman of the Council on Environmental Quality acting as the head of the Office of Environmental Quality pursuant to the Environmental Quality Improvement Act of 1970, Pub. L. 91-224, 42 U.S.C. 4371 through 4374.

[44 FR 34946, June 18, 1979, as amended at 47 FR 6277, Feb. 11, 1982]

### § 1517.3 Open meeting requirement.

(a) Every portion of every meeting of the Council is open to public observation subject to the exemptions provided in § 1517.4. Members of the Council may not jointly conduct or dispose of the business of the Council other than in accordance with this part.

(b) The Council will conduct open to public observation periodic meetings involving Council discussions of Council business including where appropriate matters outside the scope of this part. Such meetings will be noticed pursuant to § 1517.6.

(c) Members of the public may attend open meetings of the Council for the sole purpose of observation and may not participate in or photograph any meeting without prior permission of the Council. Members of the public who desire to participate in or photograph an open meeting of the Council may request permission to do so from the General Counsel of the Council before such meeting. Members of the public may record open meetings of the Council by means of any mechanical or electronic device unless the Council determines such recording would disrupt the orderly conduct of such meeting.

[44 FR 34946, June 18, 1979, as amended at 47 FR 6277, Feb. 11, 1982]

### § 1517.4 Exceptions.

(a) A meeting or portion thereof may be closed to public observation, and information pertaining to such meeting or portion thereof may be withheld from the public, if the Council determines that such meeting or portion thereof or disclosure of such information is likely to:

(1) Disclose matters that are (i) specifically authorized under criteria established by an Executive order to be kept secret in the interest of national defense or foreign policy and (ii) in fact properly classified pursuant to that Executive order;

(2) Relate solely to the internal personnel rules and practices of the Council;

(3) Disclose matters specifically exempted from disclosure by statute (other than the Freedom of Information Act, 5 U.S.C. 552), provided that the statute: (i) Requires that the matters be withheld from the public in such a manner as to leave no discre

ion on the issue, or (ii) establishes articular criteria for withholding or efers to particular types of matters to e withheld;

(4) Disclose the trade secrets and ommercial or financial information btained from a person and privileged r confidential;

(5) Involve accusing any person of a rime, or formally censuring any erson;

(6) Disclose information of a person- l nature if disclosure would constitute clearly unwarranted invasion of per- onal privacy;

(7) Disclose investigatory records ompiled for law enforcement pur- oses, or information which if written ould be contained in such records, ut only to the extent that the pro- uction of those records or informa- ion would:

(i) Interfere with enforcement pro- eedings,

(ii) Deprive a person of a right to a air trial or an impartial adjudication,

(iii) Constitute an unwarranted inva- ion of personal privacy,

(iv) Disclose the identity of a confi- ential source and, in the case of a ecord compiled by a criminal law en- orcement authority in the course of a riminal investigation, or by an agency onducting a lawful national security ntelligence investigation, confidential nformation furnished only by the onfidential source,

(v) Disclose investigative techniques and procedures, or,

(vi) Endanger the life or physical safety of law enforcement personnel;

(8) Disclose information contained in or related to examination, operating, or condition reports prepared by, on behalf of, or for the use of an agency responsible for the regulation or supervision of financial institutions;

(9) Disclose information the premature disclosure of which would be likely to significantly frustrate implementation of a proposed action of the Council. This exception shall not apply in any instance where the Council has already disclosed to the public the content or nature of the proposed action, or where the Council is required by law to make such disclosure on its own initiative prior to taking final action on the proposal; or

(10) Specifically concern the issuance of a subpoena by the Council, or the participation of the Council in a civil action or proceeding, an action in a foreign court or international tribunal, or an arbitration, or the initiation, conduct, or disposition by the Council of a particular case of formal adjudication pursuant to the procedures in 5 U.S.C. 554 or otherwise involving a determination on the record after opportunity for a hearing.

(b) Before a meeting is closed to public observation the Council shall determine whether or not the public interest requires that the meeting be open. The Council may open a meeting to public observation which could be closed under paragraph (a) of this section, if the Council finds it to be in the public interest to do so.

### § 1517.5 Procedure for closing meetings.

(a) A majority of the entire membership of the Council may vote to close to public observation a meeting or a portion or portions thereof, or to withhold information pertaining to such meeting. A separate vote of the members of the Council shall be taken with respect to each meeting of the Council, a portion or portions of which are proposed to be closed to the observation of the public or with respect to any information concerning such meetings or portion thereof. A single vote may be taken with respect to a series of meetings, a portion or portions of which are proposed to be closed to the public, or with respect to information concerning such series of meetings, so long as each meeting in such series involves the same particular matters and is scheduled to be held no more than thirty days after the initial meeting in such series. The vote of each member of the Council participating in a vote shall be recorded and no proxies shall be allowed.

(b) Whenever any person whose interest may be directly affected by a portion of a meeting requests that the Council close that portion to public observation for any of the reasons referred to in § 1517.4(a) the Council, upon request of any of the members of the Council, shall decide by recorded vote whether to close that portion of the meeting.

(c) For every meeting or portion thereof closed under this part, the General Counsel of the Council before such meeting is closed shall publicly certify that, in his or her opinion, the meeting may properly be closed to the public stating each relevant exemptive provision. The Council shall retain a copy of the General Counsel's certification, together with a statement from the presiding officer of the meeting setting forth the time and place of the meeting and listing the persons present.

(d) Within one day of any vote taken on a proposal to close a meeting, the Council shall make publicly available a record reflecting the vote of each member on the question. In addition, within one day of any vote which closes a portion or portions of a meeting to the public, the Council shall make publicly available a full written explanation of its closure action together with a list naming all persons expected to attend and identifying their affiliation, unless such disclosure would reveal the information that the meeting itself was closed to protect.

(e) Following any announcement that the Council intends to close a meeting or portion thereof, any person may make a request that the meeting or portion thereof be opened. Such request shall be made of the Chairman of the Council who shall ensure that the request is circulated to all members of the Council on the same business day on which it is received. The request shall set forth the reasons why the requestor believes the meeting should be open. The Council upon the request of any member or its General Counsel, shall vote on the request.

### § 1517.6 Notice of meetings.

(a) Except as otherwise provided in this section, the Council shall make a public announcement at least one week before a meeting, to include the following:

(1) Time, place, and subject matter of the meeting;

(2) Whether the meeting is to be open or closed; and

(3) Name and telephone number of the official who will respond to requests for information about the meeting.

(b) A majority of the members of the Council may determine by recorded vote that the business of the Council requires a meeting to be called with less than one week's notice. At the earliest practicable time, the Council shall publicly announce the time, place and subject matter of the meeting, and whether or not it is to be open or closed to the public.

(c) If announcement of the subject matter of a closed meeting would reveal the information that the meeting itself was closed to protect, the subject matter shall not be announced.

(d) Following the public announcement required by paragraph (a) or (b) of this section:

(1) A majority of the members of the Council may change the time or place of a meeting. At the earliest practicable time, the Council shall publicly announce the change.

(2) A majority of the entire membership of the Council may change the subject matter of a meeting, or the determination to open or close a meeting to the public, if it determines by a recorded vote that the change is required by the business of the Council and that no earlier announcement of the change was possible. At the earliest practicable time, the Council shall publicly announce the change, and the vote of each member upon the change.

(e) Individuals or organizations having a special interest in activities of the Council may request the Council to place them on a mailing list for receipt of information available under this section.

(f) Following public announcement of a meeting, the time or place of a meeting may be changed only if the change is announced publicly at the earliest practicable time. The subject matter of a meeting or the determination to open or close a meeting may be changed following public announcement of a meeting only if both of the following conditions are met:

(1) There must be a recorded vote of a majority of the Council that the business of the Council requires the change and that no earlier announcement of such change was possible; and

(2) There must be a public announcement of the change and of the individual Council members' votes at the earliest practicable time.

(g) Immediately following each public announcement required by this section, the following information, as applicable, shall be submitted for publication in the FEDERAL REGISTER.

(1) Notice of the time, place, and subject matter of a meeting;

(2) Whether the meeting is open or closed;

(3) Any change in one of the preceding; and

(4) The name and telephone number of the official who will respond to requests for information about the meeting.

### § 1517.7 Records of closed meetings.

(a) A record of each meeting or portion thereof which is closed to the public shall be made and retained for two years or for one year after the conclusion of any Council proceeding involved in the meeting whichever occurs later. The record of any portion of a meeting closed to the public shall be a verbatim transcript or electronic recording. In lieu of a transcript or recording, a comprehensive set of minutes may be produced if the closure decision was made pursuant to § 1517.4(a) (8) or (10).

(b) If minutes are produced, such minutes shall fully and clearly describe all matters discussed, provide a full and accurate summary of any actions taken and the reasons expressed therefor, and include a description of each of the views expressed on any item. The minutes shall also reflect the vote of each member of the Council on any roll call vote taken during the proceedings and identify all documents produced at the meeting.

(c) The following documents shall be retained by the Council as part of the transcript, recording, or minutes of the meeting:

(1) Certification by the General Counsel that the meeting may properly be closed; and

(2) Statement from the presiding officer of the meeting setting forth the date, time, and place of the meeting and listing the persons present.

(d) The Council shall make promptly available to the public at its offices at 722 Jackson Place, NW., Washington, DC the transcript, electronic recording, or minutes maintained as a record of a closed meeting, except for such information as may be withheld under one of the provisions of § 1517.5. Copies of such transcript, minutes, or transcription of an electronic recording, disclosing the identity of each speaker, shall be furnished to any person at the actual cost of duplication or transcription.

(e) [Reserved]

(f) Requests to review or obtain copies of records other than transcripts, electronic recordings or minutes of a meeting will be processed under the Freedom of Information Act (5 U.S.C. 552) or, where applicable, the Privacy Act of 1974. (5 U.S.C. 552a). Nothing in these regulations authorizes the Council to withhold from any individual any record, including the transcripts or electronic recordings described in § 1517.8, to which the individual may have access under the Privacy Act of 1974 (5 U.S.C. 552a).

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# FINDING AIDS

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A list of CFR titles, subtitles, chapters, subchapters and parts and an alphabetical list of agencies publishing in the CFR are included in the CFR Index and Finding Aids volume to the Code of Federal Regulations which is published separately and revised annually.

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# Material Approved for Incorporation by Reference

**(Revised as of June 30, 1989)**

The Director of the Federal Register has approved under 5 U.S.C. 552(a) and 1 CFR Part 51 the incorporation by reference of the following publications. This ist contains only those incorporations by reference effective as of the revision ate of this volume. Incorporations by reference found within a regulation are ffective upon the effective date of that regulation. For more information on ncorporation by reference, see the preliminary pages of this volume.

## 0 CFR CHAPTER I (PARTS 796, 799)—SUBCHAPTER R—TOXIC SUBSTANCES CONTROL ACT
## ENVIRONMENTAL PROTECTION AGENCY

| | **40 CFR** |
|---|---|
| **American Chemical Society** | |
| Copies available from: EPA TSCA Document Processing Center, Rm. G-004 Northeast Mall, 401 M Street, SW., Washington, DC 20460 | |
| Environmental Science and Technology, Vol. 14, No. 11, Prediction of the Volatilization Rates of High-Volatility Chemicals from Natural Water Bodies, Smith, et al.. | 799.1285(e)(2)(i) |
| ASTM D 1193-77 (R 83) Standard Specification for Reagent Water......... | 796.1550(b)(1)(iii);<br>796.1570(b)(1)(ii);<br>796.1720(b)(1)(ii) and (2)(i);<br>796.1840(b)(1)(ii);<br>796.1860(b)(1)(i);<br>796.3500(b)(1)(ii);<br>796.3700(b)(2)(i)(B);<br>796.37800(b)(1)(iv)(A) |
| ASTM D 2879-86 Standard Test Method for Vapor Pressure—Temperature Relationship and Initial Decomposition Temperature of Liquids by Isotoniscope. | 796.1950(b)(2)(i) |
| ASTM D 1836-83 Standard Specification for Commercial Hexanes, pp. 966-967. | 799.2155(a)(1) |
| ASTM Special Technical Publication 854, copies available from: EPA TSCA Document Processing Center, Rm. G-004 Northeast Mall, 401 M Street, SW., Washington, DC 20460. | |
| ASTM STP 854, "Aquatic Safety Assessment of Chemicals Sorbed to Sediments", by W.J. Adams et al, as published in Aquatic Toxicology and Hazard Assessment: Seventh Symposium.. | 799.500(c)(4)(i)(B);<br>799.4000(d)(3)(i) |
| ASTM STP 854, "Phoxocephalid Amphipod Bioassay for Marine Sediment Toxicity", by R.C. Swartz, et al, as published in Aquatic Toxicology and Hazard Assessment: Seventh Symposium.. | 799.500(c)(4)(i)(A) |
| Developments in Industrial Microbiology, Vol. 18, Chapter 11, 1977; An Artificial Microbiol Ecosystem for Determining Effects and Rate of Toxicants in a Salt-March Environment, A.W. Bouquin et al., copies available from: EPA TSCA Document Processing Center, Rm. G-004 Northeast Mall, 401 M Street, SW., Washington, DC 20460. | 799.4000(c)(1)(i);<br>799.1285(e)(1)(i);<br>799.500(d)(2)(i)(B) |

**40 CFR CHAPTER I (PARTS 798, 799)—SUBCHAPTER R—TOXIC SUBSTANCES CONTROL ACT—Continued**
**ENVIRONMENTAL PROTECTION AGENCY—Continued**

| | 40 CFR |
|---|---|
| Developments in Industrial Microbiology, Vol. 18, Chapter 11, 1977; An Artificial Microbial Ecosystem for Determining Effects and Fate of Toxicants in a Salt-Marsh Environment, Vol. 18, Chapter 11 (1979). | 799.4000(c)(1)(i) |
| **Halogenated Solvents Industry Alliance**<br>1612 K Street, NW., Suite 300, Washington, DC 20006 | |
| Protocol 1,1,1-Trichloroethane (TCEA): Inhalation Developmental Toxicity Probe Study in Rats. | 799.4400 |
| Protocol 1,1,1-Trichloroethane (TCEA): Inhalation Developmental Toxicity in Rats. | 799.4400 |
| Protocol 1,1,1-Trichloroethane (TCEA): Inhalation Developmental Toxicity Probe Study in Rabbits. | 799.4400 |
| Protocol 1,1,1-Trichloroethane (TCEA): Inhalation Developmental Toxicity Study in Rabbits. | 799.4400 |
| **Organization for Economic Co-operation and Development**<br>OECD Publication and Information Center, Room Number 1207, 1750 Pennsylvania Avenue, N.W., Washington, DC | |
| "Teratogenicity", Number 414, adopted May 12, 1981 | 799.1650(c) |

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# Toxic Substances Chemical—CAS Number Index

For the convenience of the user, the Office of Pesticides and Toxic Substances, Environmental Protection Agency, has developed the following Toxic Substances CAS Number/Chemical Index. Publication of this index corresponds to the information given in the CFR. For further information regarding these indices, inquiries may be made to Mary F. Travers, Federal Register Staff, Office of Pesticides and Toxic Substances, Environmental Protection Agency, 401 M St., SW., Washington DC 20460, TS-788B (202-382-2253).

EDITORIAL NOTE: This listing is provided for information purposes only.

| CAS No. | Name(s) used in regulations | 40 CFR Cites |
|---|---|---|
| 591-08-2 | Acetamide, *N*-(aminothioxomethyl)- | 716.120 |
| 3956-55-6 | Acetamide, *N*-[5-[bis[2-(acetyloxy)ethyl]amino]-2-[(2-bromo-4,6-dinitrophenyl)azo]-4-ethoxyphenyl]-. | 712.30(l), 716.120 |
| 685-91-6 | Acetamide, *N,N*-diethyl- | 716.120 |
| 60-35-5 | Acetamide (Ethanamide) | 704.225(a) |
| 640-19-7 | Acetamide, 2-fluoro- | 799.5055(c), (e)(1) |
| 25168-24-5 | Acetic acid, 2,2'-[(dibutylstannylene)bis(thio)]bis-,diisooctyl ester. | 712.30(g), 716.120 |
| 94-75-7 | Acetic acid, (2,4-dichlorophenoxy)- | 799.5055(c), (d)(2) |
| 26636-01-1 | Acetic acid, 2,2'-[(dimethylstannylene)bis(thio)]bis-,diisooctyl ester. | 712.30(g), 716.120 |
| 62-74-8 | Acetic acid, fluoro-, sodium salt | 716.120 |
| 54849-38-6 | Acetic acid, 2,2',2''-[(methylstannylidyne)tris(thio)]tris-,triisooctyl ester. | 712.30(g), 716.120 |
| 108-05-4 | Acetic acid ethenyl ester | 716.120 |
| 75-05-8 | Acetonitrile | 712.30(d), 716.120 |
| 506-96-7 | Acetyl bromide | 716.120 |
| 79-06-1 | Acrylamide | 712.30(d), 716.120 |
| 68920-70-7 | Alkanes, $C_{6-18}$' chloro- | 716.120 |
| 61788-76-9 | Alkanes, chloro- | 716.120 |
| 62-53-3 | Aniline | 716.120, 799.500 |
| 120-12-7 | Anthracene | 716.120 |
| 17418-58-5 | 9,10-Anthracenedione, 1-amino-4-hydroxy-2-phenoxy-. | 712.30(v), 716.120(a)(1) |
| 12217-79-7 | 9,10-Anthracenedione, 1,5-diaminochloro-4,8-dihydroxy-. | 712.30(v), 716.120(a)(1) |
| 128-86-9 | 2,6-Anthracenedisulfonic acid, 4,8-diamino-9,10-dihydro-1,5-dihydroxy-9,10-dioxo-. | 712.30(v), 716.120(a)(1) |
| 2861-02-1 | 2,6-Anthracenedisulfonic acid, 4,8-diamino-9,10-dihydro-1,5-dihydroxy-9,10-dioxo-, disodium salt. | 712.30(v), 716.120(a)(1) |
| 6247-34-3 | 2-Anthracenesulfonic acid, 4-[[4-(acetylamino)phenyl]amino]-1-amino-9,10-dihydro-9,10-dioxo-. | 712.30(v), 716.120(a)(1) |

| CAS No. | Name(s) used in regulations | 40 CFR Cites |
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| 6424-85-7 | 2-Anthracenesulfonic acid, 4-[[4-(acetylamino)phenyl]amino]-1-amino-9,10-dihydro-9,10-dioxo-, monosodium salt. | 712.30(v), 716.120(a)(1) |
| 84-65-1 | Anthraquinone | 712.30(m), 716.120, 704.30, 799.500 |
| 7440-36-0 | Antimony | 712.30(d), 716.120 |
| 1345-04-6 | Antimony sulfide | 712.30(d), 716.120 |
|  | Antimony trisulfide |  |
| 1309-64-4 | Antimony trioxide | 712.30(d), 716.120 |
|  | Antimony oxide |  |
| 692-42-2 | Arsine, diethyl- | 716.120 |
| 696-28-6 | Arsonous dichloride, phenyl- | 716.120 |
| 17068-78-9 | Asbestiform mineral(s) | 763, 716.120 |
| 12172-73-5 | Asbestiform mineral(s) | 763, 716.120 |
| 12001-29-5 | Asbestiform mineral(s) | 763, 716.120 |
| 1332-21-4 | Asbestiform mineral(s) | 763, 716.120 |
| 105-60-2 | *2H*-Azepin-2-one, hexahydro- | 716.120 |
| 10361-37-2 | Barium chloride | 712.30(w) |
| 62-53-3 | Benzenamine | 716.120, 799.5000 |
| 142-04-1 | Benzenamine, hydrochloride | 712.30(d) |
| 1208-52-2 | Benzenamine, 2-[(4-aminophenyl)methyl]- | 716.120 |
| 106-40-1 | Benzenamine, 4-bromo- | 712.30(d), 716.120 |
| 99-29-6 | Benzenamine, 2-bromo-6-chloro-4-nitro- | 716.120 |
| 1817-73-8 | Benzenamine, 2-bromo-4,6-dinitro- | 712.30(d), 716.120 |
| 95-51-2 | Benzenamine, 2-chloro- | 712.30(d), 716.120, 799.5000 |
| 108-42-9 | Benzenamine, 3-chloro- | 712.30(d), 716.120 |
| 106-47-8 | Benzenamine, 4-chloro- | 712.30(d), 716.120, 799.5000 |
| 141-85-5 | Benzenamine, 3-chloro-, hydrochloride | 712.30(d), 716.120 |
| 3531-19-9 | Benzenamine, 2-chloro-4,6,-dinitro- | 716.120 |
| 5388-62-5 | Benzenamine, 4-chloro-2,6-dinitro- | 712.30(d), 716.120 |
| 92-49-9 | Benzenamine, *N*-(2-chloroethyl)-*N*-ethyl- | 712.30(d) |
| 95-69-2 | Benzenamine, 4-chloro-2-methyl- | 721.462 |
| 3165-93-3 | Benzenamine, 4-chloro-2-methyl-, hydrochloride | 721.462 |
| 87-63-8 | Benzenamine, 4-chloro-6-methyl- | 721.462 |
| 121-87-9 | Benzenamine, 2-chloro-4-nitro- | 712.30(d), 716.120 |
| 6283-25-6 | Benzenamine, 2-chloro-5-nitro- | 712.30(d), 716.120 |
| 89-63-4 | Benzenamine, 4-chloro-2-nitro- | 712.30(d), 716.120 |
| 635-22-3 | Benzenamine, 4-chloro-3-nitro- | 712.30(d), 716.120 |
| 827-94-1 | Benzenamine, 2,6-dibromo-4-nitro- | 712.30(d), 716.120 |
| 608-27-5 | Benzenamine, 2,3-dichloro- | 712.30(d), 716.120 |
| 554-00-7 | Benzenamine, 2,4-dichloro- | 712.30(d), 716.120 |
| 95-82-9 | Benzenamine, 2,5-dichloro- | 712.30(d), 716.120 |
| 95-76-1 | Benzenamine, 3,4-dichloro- | 712.30(d), 716.120, 799.5000 |
| 626-43-7 | Benzenamine, 3,5-dichloro- | 712.30(d), 716.120 |
| 99-30-9 | Benzenamine, 2,6-dichloro-4-nitro- | 712.30(d), 716.120, 799.5000 |
| 97-02-9 | Benzenamine, 2,4-dinitro- | 712.30(d), 716.120, 799.5000 |
| 95-53-4 | Benzenamine, 2-methyl- | 716.120 |
| 106-49-0 | Benzenamine, 4-methyl- | 716.120 |
| 101-77-9 | Benzenamine, 4,4'-methylenebis- | 712.30(d), 716.120 |
| 101-14-4 | Benzenamine, 4,4'-methylenebis[2-chloro- (MBOCA). | 704.225(a) |

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|---|---|---|
| 88-74-4 | Benzenamine, 2-nitro- | 712.30(d), 716.120, 799.5000 |
| 99-09-2 | Benzenamine, 3-nitro- | 712.30(d), 716.120 |
| 100-01-6 | Benzenamine, 4-nitro- | 712.30(d), 716.120, 799.5000 |
| 147-82-0 | Benzenamine, 2,4,6-tribromo- | 712.30(d), 716.120 |
| 634-93-5 | Benzenamine, 2,4,6-trichloro- | 712.30(d), 716.120 |
| 2778-42-9 | Benzene, 1,3-bis(1-isocyanato-1-methylethyl)- | 716.120 |
| 108-86-1 | Benzene, bromo- | 716.120 |
| 104-51-8 | Benzene, butyl- | 716.120 |
| 108-90-7 | Benzene, chloro- | 712.30(d), 716.120, 799.1051 |
| 100-44-7 | Benzene, chloromethyl- | 712.30(d) |
| 95-49-8 | Benzene, 1-chloro-2-methyl- | 712.30(d), 716.120 |
| 106-43-4 | Benzene, 1-chloro-4-methyl- | 716.120 |
| 5216-25-1 | Benzene, 1-chloro-4-(trichloromethyl)- | 799.5055(c), (e)(1) |
| 98-56-6 | Benzene, 1-chloro-4-trifluoromethyl- | 712.30(f), 716.120 |
| 95-50-1 | Benzene, 1,2-dichloro- | 712.30(d), 716.120, 799.1052, 799.5055(c) |
| 541-73-1 | Benzene, 1,3-dichloro- | 712.30(d), 716.120 |
| 106-46-7 | Benzene, 1,4-dichloro- | 712.30(d), 716.120, 799.1052 |
| 98-87-3 | Benzene, dichloromethyl- | 712.30(d) |
| 328-84-7 | Benzene, 1,2-dichloro-4-(trifluoromethyl)- | 712.30(1), 716.120 |
| 123-61-5 | Benzene, 1,3-diisocyanato- | 716.120 |
| 104-49-4 | Benzene, 1,4-diisocyanato- | 716.120 |
| 1321-38-6 | Benzene, diisocyanatomethyl- (unspecified toluene diisocyanate). | 716.120, 704.225(a) |
| 26471-62-5 | Benzene, 1,3-diisocyanatomethyl- | 716.120, 704.225(a) |
| 91-08-7 | Benzene, 1,3-diisocyanato-2-methyl- | 716.120, 704.225(a) |
| 584-84-9 | Benzene, 2,4-diisocyanato-1-methyl- (2,4-Toluene diisocyanate). | 716.120, 704.225(a) |
| 1330-20-7 | Benzene, dimethyl- | 712.30(d) |
| 95-47-6 | Benzene, 1,2-dimethyl- | 712.30(d), 716.120 |
| 108-38-3 | Benzene, 1,3-dimethyl- | 712.30(d), 716.120 |
| 106-42-3 | Benzene, 1,4-dimethyl- | 712.30(d), 716.120 |
| 98-06-6 | Benzene, (1,1-dimethylethyl)- | 716.120 |
| 37853-59-1 | Benzene, 1,1'-[1,2-ethanediylbis(oxy)]bis[2,4,6-tribromo-. | 712.30(d) |
| 100-42-5 | Benzene, ethenyl- | 712.30(d) |
| 100-41-4 | Benzene, ethyl- | 712.30(d) |
| 25550-14-5 | Benzene, ethylmethyl- (mixed isomers) | 712.30(d), 716.120, 799.2175 |
| 611-14-3 | Benzene, 1-ethyl-2-methyl- | 716.120, 716.120(b) |
| 103-71-9 | Benzene, isocyanato- | 712.30(d) |
| 5873-54-1 | Benzene, 1-isocyanato-2-[4-isocyanatophenyl)methyl]-. | 716.120 |
| 75790-84-0 | Benzene, 2-isocyanato-4-[(4-isocyanatophenyl) methyl]-1-methyl-. | 716.120 |
| 75790-87-1 | Benzene, 1-isocyanato-2-[(4-isocyanatophenyl)thio]-. | 716.120 |
| 108-88-3 | Benzene, methyl- | 712.30(d), 716.120 |
| 26447-40-5 | Benzene, 1,1'-methylenebis[isocyanato- | 716.120 |
| 2536-05-2 | Benzene, 1,1'-methylenebis[2-isocyanato- | 716.120 |
| 101-68-8 | Benzene, 1,1'-methylenebis[4-isocyanato- | 716.120 |
| 139-25-3 | Benzene, 1,1'-methylenebis[4-isocyanato-3-methyl- | 716.120 |

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| 98-83-9 | Benzene, (1-methylethenyl)- | 716.120 |
| 99-87-6 | Benzene, 1-methyl-4-(1-methylethyl)- | 716.120 |
| 13414-54-5 | Benzene, 1-[2-methyl-2-propenyl)oxy]-2-nitro- | 712.30(g), 716.120 |
| 135-98-8 | Benzene, (1-methylpropyl)- | 716.120 |
| 98-95-3 | Benzene, nitro- | 712.30(d), 716.120 |
| 101-84-8 | Benzene, 1,1'-oxybis- | 712.30(w), 716.120 |
| 85-22-3 | Benzene, pentabromoethyl- | 712.30(m), 716.120, 721.1515 |
| 608-93-5 | Benzene, pentachloro- | 712.30(d), 716.120, 799.5055(c), (d)(2) |
| 634-66-2 | Benzene, 1,2,3,4-tetrachloro- | 712.30(d), 716.120 |
| 634-90-2 | Benzene, 1,2,3,5-tetrachloro | 712.30(d), 716.120 |
| 95-94-3 | Benzene, 1,2,4,5-tetrachloro | 712.30(d), 716.120, 799.1054, 799.5055(c), (d)(2) |
| 87-61-6 | Benzene, 1,2,3-trichloro- | 712.30(d), 716.120, 799.1053 |
| 120-82-1 | Benzene, 1,2,4-trichloro- | 712.30(d), 716.120, 799.1053 |
| 108-70-3 | Benzene, 1,3,5-trichloro- | 712.30(d), 716.120 |
| 98-07-7 | Benzene, trichloromethyl- | 712.30(d) |
| 25551-13-7 | Benzene, trimethyl- (mixed isomers) | 712.30(g), 716.120 |
| 526-73-8 | Benzene, 1,2,3-trimethyl- | 712.30(g), 716.120, 799.2175 |
| 95-63-6 | Benzene, 1,2,4-trimethyl- | 712.30(d), 716.120, 716.120(b), 799.2175 |
| 108-67-8 | Benzene, 1,3,5-trimethyl- | 712.30(g), 716.120(b) 799.2175 |
| 114-49-8 | Benzeneacetic acid, $\alpha$-(hydroxymethyl)-, 9-methyl-3-oxa-9-azatricyclo$[3.3.1.0^{2,4}]$non-7-yl ester, hydrobromide, [7(*S*)-($1\alpha$ ,$2\beta$,$4\beta$,$5\alpha$,$7\beta$)]-. | 712.30(d) |
| 6106-81-6 | Benzeneacetic acid, -(hydroxymethyl)-, 9-methyl-3-oxa-9-azatricyclo$[3.3.1.0^{2,4}]$non-7-yl ester, *N*-oxide, hydrobromide, [7(*S*)-($1\alpha$ ,$2\beta$ ,$4\beta$ ,$5\alpha$ ,$7\beta$ )]-. | 712.30(d) |
| 16532-79-9 | Benzeneacetonitrile, 4-bromo- | 799.5055(c), (d)(1), (2), (e)(1) |
| 95-54-5 | 1,2-Benzenediamine | 712.30(d), 716.120 |
| 108-45-2 | 1,3-Benzenediamine | 712.30(d), 716.120 |
| 106-50-3 | 1,4-Benzenediamine | 712.30(d), 716.120 |
| 615-28-1 | 1,2-Benzenediamine, dihydrochloride | 712.30(f), 716.120 |
| 541-69-5 | 1,3-Benzenediamine, dihydrochloride | 712.30(f), 716.120 |
| 624-18-0 | 1,4-Benzenediamine, dihydrochloride | 712.30(d), 716.120 |
| 62654-17-5 | 1,4-Benzenediamine, ethanedioate (1:1) | 712.30(f), 716.120 |
| 541-70-8 | 1,3-Benzenediamine, sulfate (1:1) | 712.30(f), 716.120 |
| 16245-77-5 | 1,4-Benzenediamine, sulfate (1:1) | 712.30(f), 716.120 |
| 3663-23-8 | 1,2-Benzenediamine, 4-butyl- | 712.30(f), 716.120 |
| 95-83-0 | 1,2-Benzenediamine, 4-chloro- | 712.30(f), 716.120 |
| 5131-60-2 | 1,3-Benzenediamine, 4-chloro- | 712.30(f), 716.120 |
| 615-46-3 | 1,4-Benzenediamine, 2-chloro-, dihydrochloride | 712.30(f), 716.120 |
| 68459-98-3 | 1,2-Benzenediamine, 4-chloro-, sulfate (1:1) | 712.30(f), 716.120 |
| 68239-80-5 | 1,3-Benzenediamine, 4-chloro-, sulfate | 712.30(f), 716.120 |
| 6219-71-2 | 1,4-Benzenediamine, 2-chloro-, sulfate | 712.30(f), 716.120 |
| 42389-30-0 | 1,2-Benzenediamine, 5-chloro-3-nitro- | 712.30(f), 716.120 |
| 20103-09-7 | 1,4-Benzenediamine, 2,5-dichloro- | 712.30(f), 716.120 |
| 1197-37-1 | Benzenediamine, 4-ethoxy- | 712.30(f), 716.120 |

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| 67801-06-3 | 1,3-Benzenediamine, 4-ethoxy-, dihydrochloride | 712.30(f), 716.120 |
| 68015-98-5 | 1,3-Benzenediamine, 4-ethoxy-, sulfate (1:1) | 712.30(f), 716.120 |
| 68966-84-7 | 1,3-Benzenediamine, *ar*-ethyl-*ar*-methyl- | 712.30(f), 716.120 |
| 615-05-4 | 1,3-Benzenediamine, 4-methoxy- | 712.30(d), 716.120 |
| 5307-02-8 | 1,4-Benzenediamine, 2-methoxy- | 712.30(f), 716.120 |
| 614-94-8 | 1,3-Benzenediamine, 4-methoxy-, dihydrochloride | 712.30(f), 716.120 |
| 6219-67-6 | 1,3-Benzenediamine, 4-methoxy-, sulfate | 712.30(f), 716.120 |
| 39156-41-7 | 1,3-Benzenediamine, 4-methoxy-, sulfate (1:1) | 712.30(f), 716.120 |
| 25376-45-8 | Benzenediamine, *ar*-methyl- | 712.30(f), 716.120 |
| 2687-25-4 | 1,2-Benzenediamine, 3-methyl- | 712.30(d), 716.120 |
| 496-72-0 | 1,2-Benzenediamine, 4-methyl- | 712.30(d), 716.120 |
| 823-40-5 | 1,3-Benzenediamine, 2-methyl- | 712.30(d), 716.120 |
| 95-80-7 | 1,3-Benzenediamine, 4-methyl- | 712.30(f), 716.120 |
| 108-71-4 | 1,3-Benzenediamine, 5-methyl- | 712.30(f), 716.120 |
| 95-70-5 | 1,4-Benzenediamine, 2-methyl- | 712.30(f), 716.120 |
| 615-45-2 | 1,4-Benzenediamine, 2-methyl-, dihydrochloride | 712.30(f), 716.120 |
| 6369-59-1 | 1,4-Benzenediamine, 2-methyl-, sulfate | 712.30(d), 716.120 |
| 615-50-9 | 1,4-Benzenediamine, 2-methyl-, sulfate (1:1) | 712.30(f), 716.120 |
| 99-56-9 | 1,2-Benzenediamine, 4-nitro- | 712.30(d), 716.120 |
| 5131-58-8 | 1,3-Benzenediamine, 4-nitro- | 712.30(f), 716.120 |
| 5042-55-7 | 1,3-Benzenediamine, 5-nitro- | 712.30(f), 716.120 |
| 6219-77-8 | 1,2-Benzenediamine 4-nitro-, dihydrochloride | 712.30(f), 716.120 |
| 68239-82-7 | 1,2-Benzenediamine, 4-nitro-, sulfate (1:1) | 712.30(f), 716.120 |
| 5307-14-2 | 1,4-Benzenediamine, 2-nitro- | 712.30(f), 716.120 |
| 18266-52-9 | 1,4-Benzenediamine, 2-nitro-, dihydrochloride | 712.30(f), 716.120 |
| 68239-83-8 | 1,4-Benzenediamine, 2-nitro-, sulfate (1:1) | 712.30(f), 716.120 |
| 117-81-7 | 1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester | 712.30(d), 716.120, 799.5000 |
| 6422-86-2 | 1,4-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester | 712.30(g), 716.120 |
| 131-15-7 | 1,2-Benzenedicarboxylic acid, bis(1-methylheptyl) ester. | 716.120 |
| 84-69-5 | 1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester. | 716.120 |
| 7195-45-1 | 1,2-Benzenedicarboxylic acid, bis(oxiranylmethyl) ester. | 716.120 |
| 84-64-0 | 1,2-Benzenedicarboxylic acid, butyl cyclohexyl ester | 712.30(d), 716.120 |
| 84-78-6 | 1,2-Benzenedicarboxylic acid, butyl octyl ester | 716.120 |
| 85-68-7 | 1,2-Benzenedicarboxylic acid, butyl phenylmethyl ester. | 712.30(d), 716.120 |
| 25724-58-7 | 1,2-Benzenedicarboxylic acid, decyl hexyl ester | 716.120 |
| 119-07-3 | 1,2-Benzenedicarboxylic acid, decyl octyl ester | 712.30(d), 716.120 |
| 84-74-2 | 1,2-Benzenedicarboxylic acid, dibutyl ester | 712.30(d), 716.120, 799.5000 |
| 68515-42-4 | 1,2-Benzenedicarboxylic acid, di-$C_{7-11}$-branched and linear alkyl esters. | 799.5000 |
| 68515-49-1 | 1,2-Benzenedicarboxylic acid, di-$C_{9-11}$-branched alkyl esters, $C_{10}$-rich. | 799.5000 |
| 68515-47-9 | 1,2-Benzenedicarboxylic acid, di-$C_{11-14}$-branched alkyl esters, $C_{13}$-rich. | 799.5000 |
| 84-61-7 | 1,2-Benzenedicarboxylic acid, dicyclohexyl ester | 712.30(d), 716.120 |
| 84-66-2 | 1,2-Benzenedicarboxylic acid, diethyl ester | 712.30(d), 716.120 |
| 84-75-3 | 1,2-Benzenedicarboxylic acid, dihexyl ester | 716.120, 799.5000 |
| 68515-50-4 | 1,2-Benzenedicarboxylic acid, dihexyl ester, branched and linear. | 799.5000 |
| 26761-40-0 | 1,2-Benzenedicarboxylic acid, diisodecyl ester | 712.30(d), 716.120, 799.5000 |

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| 28553-12-0 | 1,2-Benzenedicarboxylic acid, diisononyl ester | 716.120 |
| 27554-26-3 | 1,2-Benzenedicarboxylic acid, diisooctyl ester | 716.120 |
| 131-11-3 | 1,2-Benzenedicarboxylic acid, dimethyl ester | 712.30(d), 716.120, 799.5000 |
| 84-76-4 | 1,2-Benzenedicarboxylic acid, dinonyl ester | 716.120 |
| 117-84-0 | 1,2-Benzenedicarboxylic acid, dioctyl ester | 712.30(d), 716.120 |
| 131-17-9 | 1,2-Benzenedicarboxylic acid, di-2-propenyl ester | 712.30, 716.120 |
| 119-06-2 | 1,2-Benzenedicarboxylic acid, ditridecyl ester | 712.30(d), 716.120, 799.5000 |
| 3648-20-2 | 1,2-Benzenedicarboxylic acid, diundecyl ester | 716.120, 799.5000 |
| 85-69-8 | 1,2-Benzenedicarboxylic acid, 2-ethylhexyl ester | 716.120 |
| 89-13-4 | 1,2-Benzenedicarboxylic acid, 2-ethylhexyl-8-methylnonyl ester. | 716.120 |
| 61702-81-6 | 1,2-Benzenedicarboxylic acid, hexyl isodecyl ester | 716.120 |
| 61886-60-0 | 1,2-Benzenedicarboxylic acid, isodecyl tridecyl ester | 716.120 |
| 1477-55-0 | 1,3-Benzenedimethanamine | 712.30(f) |
| 123-31-9 | 1,4-Benzenediol | 712.30(d), 716.120, 799.2200 |
| 122-09-8 | Benzeneethanamine, ,α,α-dimethyl- | 716.120 |
| 121-47-1 | Benzenesulfonic acid, 3-amino- | 716.120 |
| 16110-89-7 | Benzenesulfonic acid, 4[(4,6-dichloro-1,3,5-triazin-2-yl) amino]-. | 712.30(d) |
| 98-09-9 | Benzenesulfonyl chloride | 716.120 |
| 108-98-5 | Benzenethiol | 716.120 |
| 3319-31-1 | 1,2,4-Benzenetricarboxylic acid, · tris(2-ethylhexyl) ester. | 712.30(g), 716.120 |
| 13414-55-6 | Benzofuran, 2,3-dihydro-2,2-dimethyl-7-nitro- | 712.30(g), 716.120 |
| 68298-46-4 | 7-Benzofuranamine, 2,3-dihydro-2,2-dimethyl- | 712.30(g), 716.120 |
| 6739-62-4 | Benzoic acid, 2-[[2-amino-6-[[4'-[(3-carboxy-4-hydroxyphenyl)azo]-3,3'-dimethoxy[1,1'-biphenyl]-4-yl]azo]-5-hydroxy-7-sulfo-1-naphthalenyl]azo].<br>-5-nitro-, trisodium salt | 716.120 |
| 2429-84-7 | Benzoic acid, 5-[[4'-[(2-amino-8-hydroxy-6-sulfo-1-naphthalenyl)azo][1,1'-biphenyl]-4-yl] azo)-2-hydroxy-, disodium salt. | 716.120 |
| 2429-82-5 | Benzoic acid, 5-[[4'-[(7-amino-1-hydroxy-3-sulfo-2-naphthalenyl)azo][1,1'-biphenyl]-4-yl] azo]-2-hydroxy-, disodium salt. | 716.120 |
| 2429-79-0 | Benzoic acid, 5-[[4'-[(1-amino-4-sulfo-2-naphthalenyl)azo][1,1'-biphenyl]-4-yl] azo]-2-hydroxy-, disodium salt. | 716.120 |
| 2905-65-9 | Benzoic acid, 3-chloro-, methyl ester | 712.30(d) |
| 2429-81-4 | Benzoic acid, 5-[[4'-[[2,6-diamino-3-[[8-hydroxy-3,6-disulfo-7-[(4-sulfo-1-naphthalenyl)azo]-2-naphthalenyl]azo]-5-methylphenyl]azo] [1,1'-biphenyl]-4-yl] azo]-2-hydroxy-, tetrasodium salt. | 716.120 |
| 6637-88-3 | Benzoic acid, 5-[[4'-[(2,6-diamino-3-methyl-5-sulfophenyl)azo]-3,3'-dimethyl] [1,1'-biphenyl]-4-yl]azo]-2-hydroxy-, disodium salt. | 716.120 |
| 2586-58-5 | Benzoic acid, 5-[[4'-[[2,6-diamino-3-methyl-5-[(4-sulfophenyl)azo]phenyl]azo][1,1'-biphenyl]-4-yl]azo]-2-hydroxy-, disodium salt. | 716.120 |
| 6360-54-9 | Benzoic acid, 5-[[4'-[[2,6-diamino-3-methyl-5-[(4-sulfophenyl)azo]phenyl]azo][1,1'-biphenyl]-4-yl]azo]-2-hydroxy-3-methyl-, disodium salt. | 716.120 |

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| 2893-80-3 | Benzoic acid, 5-[[4'-[[2,4-dihydroxy-3-[(4-sulfophenyl)azo]phenyl]azo][1,1'-biphenyl]-4-yl]azo]-2-hydroxy-, disodium salt. | 716.120 |
| 8014-91-3 | Benzoic acid, 3,3'-[(3,7-disulfo-1,5-naphthalenediyl)bis[azo(6-hydroxy-3,1-phenylene) azo[6(or 7)-sulfo-4,1-naphthalenediyl] azo[1,1'biphenyl]-4,4'-diylazo]] bis[6-hydroxy-, hexasodium salt. | 716.120 |
| 61386-02-5 | Benzoic acid, 3,3'-methylenebis[6-amino-di-2-propenyl] ester. | 721.205 |
| 95-14-7 | 1*H*-Benzotriazole | 712.30(w), 716.120 |
| 29385-43-1 | 1*H*-Benzotriazole, methyl- | 712.30, 716.120 |
| 98-88-4 | Benzoyl chloride | 712.30(d) |
| 120-32-1 | 2-Benzyl-4-chlorophenol | 712.30, 716.120 |
| 90-42-6 | [1,1'-Bicyclohexyl]-2-one | 716.120 |
| 1464-53-5 | 2,2'-Bioxirane | 712.30(d), 716.120 |
| 92-52-4 | 1,1'-Biphenyl | 712.30(d), 716.120, 799.925 |
| 2052-07-5 | 1,1'-Biphenyl, 2-bromo- | 721.600 |
| 2113-57-7 | 1,1'-Biphenyl, 3-bromo- | 721.600 |
| 92-66-0 | 1,1'-Biphenyl, 4-bromo- | 721.600 |
| 13654-09-6 | 1,1'-Biphenyl, 2,2',3,3',4,4',5,5',6,6'-decabromo- | 721.600 |
| 92-86-4 | 1,1'-Biphenyl, 4,4'-dibromo- | 721.600 |
| 91-97-4 | 1,1'-Biphenyl, 4,4'-diisocyanato-3,3'-dimethyl- | 716.120 |
| 36355-01-8 | 1,1'-Biphenyl, hexabromo- | 721.600 |
| 27753-52-2 | 1,1'-Biphenyl, nonabromo- | 721.600 |
| 27858-07-7 | 1,1'-Biphenyl, octabromo- | 721.600 |
| 20282-70-6 | [1,1'-Biphenyl]-4,4'-bis(diazonium), 3,3'-dimethoxy- | 716.120 |
| 92-87-5 | [1,1'-Biphenyl]-4,4'-diamine | 716.120 |
| 1331-47-1 | [1,1'-Biphenyl]-4,4'-diamine, dichloro- | 716.120 |
| 580-51-8 | [1,1'-Biphenyl]-3-ol | 716.120 |
| 92-69-3 | [1,1'-Biphenyl]-4-ol | 716.120 |
| 111-91-1 | Bis(2-chloroethoxy)methane | 716.120, 799.5055(c), (d)(2), (e)(1) |
| 108-60-1 | Bis(2-chloroisopropyl) ether | 716.120, 799.5055(c), (d)(2) |
| 80-05-7 | Bisphenol A | 712.30(k), 716.120, 799.940 |
| 16532-79-9 | 4-Bromobenzyl cyanide | 799.5055(c) |
| 92-66-0 | Bromobiphenyl | 721.600 |
| 2052-07-5 | 2-Bromobiphenyl | 721.600 |
| 2113-57-7 | 3-Bromobiphenyl | 721.600 |
| 75-25-2 | Bromoform | 716.120, 799.5055(c), (d)(2) |
| 7726-95-6 | Bromine | 712.30(w) |
| 126-99-8 | 1,3-Butadiene, 2-chloro- | 712.30(d), 712.30(m), 716.120 |
| 87-68-3 | 1,3-Butadiene, 1,1,2,3,4,4-hexachloro- | 712.30(d), 716.120 |
| 91-96-3 | Butanamide, *N,N'*-(3,3'-dimethyl[1,1'-biphenyl]-4,4'-diyl)bis[3-oxo-. | 716.120 |
| 111-92-2 | 1-Butanamine, *N*-butyl- | 716.120 |
| 78-93-3 | 2-Butanone | 712.30(d), 716.120 |
| 96-29-7 | 2-Butanone, oxime | 712.30(d), 716.120 |
| 4170-30-3 | 2-Butenal | 716.120, 712.30(w) |
| 25168-21-2 | 2-Butenoic acid, 4,4'-[(dibutylstannylene)bis(oxy)]bis[4-oxo- ,diisoctyl ester, (*Z,Z*)-. | 712.20(g), 716.120 |

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|---|---|---|
| 124-17-4 | 2-(2-Butoxyethoxy)ethyl acetate | 712.30(j), 716.120 |
| 939-97-9 | *p-tert*-Butylbenzaldehyde | 704.33, 716.120 |
| 98-73-7 | *p-tert*-Butylbenzoic acid | 704.33, 716.120 |
| 98-51-1 | *p-tert*-Butyltoluene | 704.33, 716.120 |
| 10401-50-0 | C.I. Direct Blue 218 | 716.120 |
| 68309-98-8 | Cadmate (6-), [[[1,2-ethanediylbis[nitrilobis (methylene)]]tetrakis[phosphonato]](8-)-*N*,*N*',*O*,*O*',*O*''',*O*''''']-, pentapotassium hydrogen, (*OC*-6-21)-. | 704.95 |
| 61789-36-4 | Calcium naphthenate | 712.30(h), 716.120 |
| 86-74-8 | 9*H*-Carbazole | 716.120 |
| 7782-50-5 | Chlorine | 704.225(a) |
| 95-51-2 | 2-Chloroaniline | 716.120, 799.5000 |
| 106-47-2 | 4-Chloroaniline | 716.120, 799.5000 |
| 5216-25-1 | 4-Chlorobenzotrichloride | 799.5055(c), (e)(1) |
| 126-99-8 | Chloroprene | 712.30(d), 712.30(m), 716.120 |
| 88-04-0 | *p*-Chloro-*m*-xylenol | 712.30, 716.120 |
| 7440-48-4 | Cobalt | 716.120 |
| 61789-51-3 | Cobalt naphthenate | 712.30(h), 716.120 |
| 68025-39-8 | Cobaltate (6-), [[[1,2-ethanediylbis[nitrilobis (methylene)]]tetrakis[phosphonato]](6-)-*N*,*N*',*O*,*O*',*O*''',*O*''''']-, pentaammonium hydrogen, (*OC*-6-21)-. | 704.95 |
| 67924-23-6 | Cobaltate (6-), [[[1,2-ethanediylbis[nitrilobis (methylene)]]tetrakis[phosphonato]](8-)-*N*,*N*',*O*,*O*',*O*''',*O*''''']-, pentapotassium hydrogen, (*OC*-6-21)-. | 704.95 |
| 67969-67-9 | Cobaltate (6-), [[[1,2-ethanediylbis[nitrilobis (methylene)]]tetrakis[phosphonato]](8-)-*N*,*N*',*O*,*O*',*O*''',*O*''''']-, pentasodium hydrogen, (*OC*-6-21)-. | 704.95 |
| 10124-43-3 | Cobaltous sulfate | 712.30(w) |
| 95-48-7 | *o*-Cresol | 712.30(d), 716.120, 799.1250 |
| 108-39-4 | *m*-Cresol | 712.30(d), 716.120, 799.1250 |
| 106-44-5 | *p*-Cresol | 712.30(d), 716.120, 799.1250 |
| 98-82-8 | Cumene | 712.30(m), 716.120 |
| 16143-79-6 | Cuprate(4-), [ μ-[[6,6'-[3,3'-dihydroxy[1,1'-biphenyl]-4-4'-diyl)bis(azo)]bis[4-amino-5-hydroxy-1,3-naphthalenedisulfonato]](8-)]]di-,tetrasodium | 716.120 |
| 6656-03-7 | Cuprate(3-, [ μ-[7-([3,3'-dihydroxy-4'-[[1-hydroxy-6-(phenylamino)-3-sulfo-2-naphthalenyl] azo][1,1'-biphenyl]-4-yl]azo]-8-hydroxy-1,6-naphthalenedisulfonato(7-)]]di-, trisodium. | 716.120 |
| 16071-86-6 | Cuprate(2-), [5-[[4'-[[2,6-dihydroxy-3-[(2-hydroxy-5-sulfophenyl)azo]phenyl]azo][1,1'-biphenyl]-4-yl]azo]-2-hydroxybenzonato(4-)]-, disodium. | 716.120 |
| 67989-89-3 | Cuprate (6-), [[[1,2-ethanediylbis[nitrilobis (methylene)]]tetrakis[phosphonato]](8-)*N*,*N*',*O*,*O*',*O*''',*O*''''']-, pentapotassium hydrogen,(*OC*-6-21)-. | 704.95 |
| 25637-99-4 | Cyclodecane, hexabromo- | 712.30(d) |

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|---|---|---|
| 3194-55-6 | Cyclododecane, 1,2,5,6,9,10-hexabromo- | 712.30(d) |
| 106-51-4 | 2,5-Cyclohexadiene-1,4-dione | 712.30(d), 716.120 |
| 110-82-7 | Cyclohexane | 712.30(p), 716.120 |
| 14228-73-0 | Cyclohexane, 1,4-bis[(2,3-epoxypropoxy)methyl]- | 716.120 |
| 38661-72-2 | Cyclohexane, 1,3-bis(isocyanatomethyl)- | 716.120 |
| 10347-54-3 | Cyclohexane, 1,4-bis(isocyanatomethyl)- | 716.120 |
| 3322-93-8 | Cyclohexane, 1,2-dibromo-4-(1,2-dibromoethyl)- | 712.30(k), 716.120 |
| 2556-36-7 | Cyclohexane, 1,4-diisocyanato- | 716.120 |
| 4098-71-9 | Cyclohexane, 5-isocyanato-1-(isocyanatomethyl)-1,3,3-trimethyl-. | 716.120 |
| 5124-30-1 | Cyclohexane, 1,1'-methylenebis[4-isocyanato- | 716.120 |
| 5493-45-8 | 1,2-Cyclohexanedicarboxylic acid, bis(oxiranylmethyl) ester. | 716.120 |
| 108-94-1 | Cyclohexanone | 712.30(d), 716.120 |
| 100-40-3 | Cyclohexene, 4-ethenyl- | 712.30(w) |
| 78-59-1 | 2-Cyclohexen-1-one, 3,5,5-trimethyl- | 712.30(d), 716.120 |
| 77-47-4 | 1,3-Cyclopentadiene, 1,2,3,4,5,5-hexachloro- | 712.30(d), 716.120 |
| 94-75-7 | 2,4-D | 799.5055(c) |
| 13654-09-6 | Decabromobiphenyl | 721.230 |
| 131-17-9 | Diallyl phthalate | 712.30, 716.120 |
| 5307-02-8 | p-Diaminoanisole | 712.30(f), 716.120 |
| 39156-41-7 | 2,4-Diaminoanisole sulfate | 712.30(f), 716.120 |
| 74-95-3 | Dibromomethane | 799.5055(c), (d)(2) |
| 92-86-4 | Dibromobiphenyl | 721.230 |
| 128-39-2 | 2,6-Di-*tert*-butyl phenol | 712.30(p), 716.120 |
| 84-74-2 | Di-*n*-butyl phthalate | 712.30(d), 716.120, 799.5000 |
| 25168-21-2 | Dibutyltin bis(isooctyl maleate) | 712.30(g), 716.120 |
| 25168-24-5 | Dibutyltin *S,S'*-bis(isooctyl mercaptoacetate) | 712.30(g), 716.120 |
| 1185-81-5 | Dibutyltin bis(lauryl mercaptide) | 712.30(g), 716.120 |
| 77-58-7 | Dibutyltin dilaurate | 712.30(g), 716.120 |
| 95-76-1 | 3,4-Dichloroaniline | 712.30(d), 716.120, 799.5000 |
| 95-50-1 | 1,2-Dichlorobenzene | 712.30(d), 716.120, 799.1052, 799.5055(c), (d)(2) |
| 328-84-7 | Dichlorobenzotrifluoride | 712.30(1), 716.120 |
| 75-34-3 | 1,1-Dichloroethane | 716.120, 799.5055(c), (d)(2) |
| 75-43-4 | Dichloromonofluoromethane | 712.30(w), 716.120 |
| 99-30-9 | 2,6-Dichloro-4-nitroaniline | 712.30(d), 716.120, 799.5000 |
| 96-23-1 | 1,3-Dichloropropanol | 799.5055(c), (d)(1), (e)(1) |
| 111-42-2 | Diethanolamine | 712.30(w), 716.120 |
| 124-17-4 | Diethylene butyl ether acetate | 799.1560 |
| 112-34-5 | Diethylene glycol butyl ether | 799.1560 |
| 111-90-0 | Diethylene glycol monoethyl ether | 712.30, 716.120 |
| 111-77-3 | Diethylene glycol monomethyl ether | 712.30, 716.120 |
| 117-81-7 | Di-2-ethylhexyl phthalate | 712.39(d), 716.120, 799.5000 |
| 68515-42-4 | Di(heptyl-, nonyl-, undecyl-) phthalate (mixed isomers). | 799.5000 |
| 84-75-3 | Di-*n*-hexyl phthalate | 716.120, 799.5000 |
| 68515-50-4 | Dihexyl phthalate (mixed isomers) | 799.5000 |
| 94-58-6 | Dihydrosafrole | 799.5055(c), (d)(2) |

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|---|---|---|
| 25550-98-5 | Diisodecyl phenyl phosphite | 712.30(p), 716.120, 799.5000 |
| 26761-40-0 | Diisodecyl phthalate | 712.30(d), 716.120, 799.5000 |
| 68515-49-1 | Diisodecyl phthalate (mixed isomers) | 799.5000 |
| 69009-90-1 | Diisopropyl biphenyl | 712.30(k), 716.120 |
| 357-57-3 | 2,3-Dimethoxystrychnidin-10-one | 716.120 |
| 68-12-2 | Dimethyl formamide | 712.30(w), 716.120 |
| 131-11-3 | Dimethyl phthalate | 712.30(d), 716.120, 799.5000 |
| 26636-01-1 | Dimethyltin *S,S'*-bis(isooctyl mercaptoacetate) | 712.30(g), 716.120 |
| 97-02-9 | 2,4-Dinitroaniline | 712.30(d), 716.120, 799.5000 |
| 828-00-2 | 1,3-Dioxan-4-o1, 2,6-dimethyl-, acetate | 716.120 |
| 646-06-0 | 1,3-Dioxolane | 712.30(g), 716.120 |
| 34590-94-8 | Dipropylene glycol monomethyl ether | 712.30(w), 716.120 |
| 126-80-7 | Disiloxane, 1,1,3,3-tetramethyl-1,3-bis-[3-oxiranylmethoxy)propyl]-. | 716.120 |
| 624-92-0 | Disulfide, dimethyl- | 704.225(a) |
| 119-06-2 | Ditridecyl phthalate | 712.30(d), 716.120, 799.5000 |
| 68515-47-9 | Ditridecyl phthalate (mixed isomers) | 799.5000 |
| 3648-20-2 | Diundecyl phthalate | 716.120, 799.5000 |
| 72-20-8 | Endrin | 799.5055(c), (d)(2) |
| 75-04-7 | Ethanamine | 716.120 |
| 121-44-8 | Ethanamine, *N,N*-diethyl- | 716.120 |
| 122-07-6 | Ethanamine, 2,2-dimethoxy-*N*-methyl- | 712.30(d) |
| 109-89-7 | Ethanamine, *N*-ethyl- | 716.120 |
| 107-04-4 | Ethane, 1-bromo-2-chloro- | 721.648 |
| 75-00-3 | Ethane, chloro- | 712.30(w), 716.120 |
| 75-68-3 | Ethane, 1-chloro-1,1-difluoro- | 712.30(w), 716.120 |
| 75-34-3 | Ethane, 1,1-dichloro- | 716.120, 799.5055(c) |
| 107-06-2 | Ethane, 1,2-dichloro- | 716.120 |
| 75-37-6 | Ethane, 1,1-difluoro- | 712.30(w), 716.120 |
| 534-15-6 | Ethane, 1,1-dimethoxy- | 712.30(d) |
| 111-91-1 | Ethane, 1,1'-[methylenebis(oxy)]bis[2-chloro- | 716.120, 799.5055(c) |
| 79-24-3 | Ethane, nitro- | 712.30(w), 716.120 |
| 79-27-6 | Ethane, 1,1,2,2-tetrabromo- | 704.225(a) |
| 630-20-6 | Ethane, 1,1,1,2-tetrachloro- | 716.120 |
| 79-34-5 | Ethane, 1,1,2,2-tetrachloro- | 716.120 |
| 71-55-6 | Ethane, 1,1,1-trichloro- | 712.30(d), (w), 716.120, 799.3450 |
| 79-00-5 | Ethane, 1,1,2-trichloro- | 712.30, 716.120 |
| 76-13-1 | Ethane, 1,1,2-trichloro-1,2,2-trifluoro- | 716.120 |
| 111-40-0 | 1,2-Ethanediamine, *N*-(2-aminoethyl)- | 712.30(d), 716.120, 799.1575 |
| 111-76-2 | Ethanol, 2-butoxy- | 712.30, 716.120 |
| 115-96-8 | Ethanol, 2-chloro-, phosphate (3:1) (Tris(2-chloroethyl) phosphate). | 704.225(a) |
| 66422-95-5 | Ethanol, 2-(2,4-diaminophenoxy)-, dihydrochloride | 712.30(f), 716.120 |
| 24442-57-7 | Ethanol, 1,2-dibromo-, acetate | 712.30(d) |
| 111-21-7 | Ethanol, 2,2'-[1,2-ethanediylbis(oxy)]bis-, diacetate | 712.30(j), 716.120 |
| 111-90-0 | Ethanol, 2-(2-ethoxyethoxy)- | 712.30, 716.120 |
| 111-42-2 | Ethanol, 2,2'-iminobis- | 712.30, 716.120 |
| 111-77-3 | Ethanol, 2-(2-methoxyethoxy)- | 712.30, 716.120 |
| 102-71-6 | Ethanol, 2,2',2''-nitrilotris- | 712.30, 716.120 |

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|---|---|---|
| 593-60-2 | Ethene, bromo- | 712.30(d) |
| 110-75-8 | Ethene, (2-chloroethoxy)- | 716.120 |
| 75-38-7 | Ethene, 1,1-difluoro- | 712.30(d), 716.120 |
| 75-02-5 | Ethene, fluoro- | 712.30(f), 716.120 |
| 127-18-4 | Ethene, tetrachloro- | 716.120 |
| 116-14-3 | Ethene, tetrafluoro- | 712.30(f), 716.120, 799.1700(a)(1) |
| 97-63-2 | Ethyl methacrylate | 712.30(d), 799.5055(c), (d)(2) |
| 79771-09-8 | Ethylamine, distillation residues | 721.1250 |
| 111-21-7 | Ethylene bisoxyethylene diacetate | 712.30(j), 716.120 |
| 611-14-3 | *o*-Ethyltoluene | 716.120, 716.120(b) |
| 620-14-4 | *m*-Ethyltoluene | 716.120, 716.1203 |
| 622-96-8 | *p*-Ethyltoluene | 716.120, 716.1203 |
| 12604-53-4 | Ferromanganese | 712.30(w) |
| 75-12-7 | Formamide | 712.30(d), 716.120 |
| 68-12-2 | Formamide, *N,N*-dimethyl- | 712.30, 716.120 |
| 98-01-1 | 2-Furancarboxaldehyde | 716.120 |
| 108-31-6 | 2,5-Furandione | 712.30(d), 716.120 |
| 556-52-5 | Glycidol | 712.30(d), 716.120 |
| 428-25-8 | Haloalkyl epoxide | 716.120 |
| 3389-71-7 | 1,2,3,4,7,7-Hexachloronorbornadiene | 712.30(j), 716.120, 721.342, |
| 428-59-1 | Hexafluoropropylene oxide | 712.30(d), 716.120, 721.1175 |
| 680-31-9 | Hexamethylphosphoramide | 721.350 |
| 110-54-3 | *n*-Hexane | 799.2155 |
| 822-06-0 | Hexane, 1,6-diisocyanato- | 716.120, 712.30(w) |
| 16938-22-0 | Hexane, 1,6-diisocyanato-2,2,4-trimethyl- | 716.120 |
| 15646-96-5 | Hexane, 1,6-diisocyanato-2,4,4-trimethyl- | 716.120 |
| 4835-11-4 | 1,6-Hexanediamine, *N,N'*-dibutyl- | 712.30(d) |
| 111-69-3 | Hexanedinitrile | 716.120 |
| 149-57-5 | Hexanoic acid, 2-ethyl- | 712.30(k), 716.120, 799.1650 |
| 104-76-7 | 1-Hexanol, 2-ethyl- | 716.120 |
| 530-50-7 | Hydrazine, 1,1-diphenyl- | 716.120 |
| 122-66-7 | Hydrazine, 1,2-diphenyl- | 716.120 |
| 57-56-7 | Hydrazinecarboxamide | 704.225(a) |
| 563-44-7 | Hydrazinecarboxamide, monohydrochloride (Semicarbazide hydrochloride).. | 704.225(a) |
| 74-90-8 | Hydrocyanic acid | 712.30(w) |
| 7664-39-3 | Hydrogen fluoride (Hydrofluoric acid) | 712.30(w) |
| 80-15-9 | Hydroperoxide, 1-methyl-1-phenylethyl- | 716.120 |
| 123-31-9 | Hydroquinone | 712.30(d), 716.120, 799.2200 |
| 5470-11-1 | Hydroxylamine, hydrochloride (Hydroxylammonium chloride). | 704.225 |
| 7803-49-8 | Hydroxylamine (Oxammonium) | 704.225 |
| 10046-00-1 | Hydroxylamine, sulfate (1:1) (Hydroxylamine acid sulfate). | 704.225 |
| 10039-54-0 | Hydroxylamine, sulfate (2:1) (Hydroxylamine acid sulfate). | 704.225 |
| 1000-82-4 | Hydroxymethyl urea | 712.30(h), 716.120 |
| 32568-89-1 | 2,4-Imidazolidinedione, 5,5-dimethyl-3-[2-(oxiranylmethoxy)propyl]-1-(oxiranylmethyl)-. | 716.120 |

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|---|---|---|
| 38304-52-8 | 2,4-Imidazolidenedione, 3,3'-[2-oxiranylmethoxy)-1,3-propanediyl]bis[5,5-dimethyl-1-(oxiranylmethyl)-. | 716.120 |
| 68122-86-1 | Imidazolium compounds, 4,5-dihydro-1-methyl-2-nortallow alkyl-1-(2-tallow amidoethyl), methyl sulfates. | 712.30(w), 716.120(a)(1) |
| 85-44-9 | 1,3-Isobenzofurandione | 799.5055(c) |
| 9016-87-9 | Isocyanic acid, polymethylenepolyphenylene ester | 716.120 |
| 32052-51-0 | Isocyanic acid, trimethylcyclohexyl ester | 716.120 |
| 5455-98-1 | 1*H*-Isoindole-1,3(2*H*)-dione, 2-(oxiranylmethyl)- | 712.30(d) |
| 78-59-1 | Isophorone | 712.30(d), 716.120 |
| 79771-08-7 | Isopropylamine, distillation residues | 721.1250 |
| 25640-78-2 | Isopropyl biphenyl | 712.30(k), 716.120 |
| 25168-06-3 | Isopropyl phenol | 712.30, 716.120 |
| 26530-20-1 | 3(2*H*)-Isothiazolone, 2-octyl- | 716.120 |
| 2763-96-4 | Isoxazolone, 5-(aminomethyl)- | 716.120 |
| 61790-14-5 | Lead naphthenate | 712.30(h), 716.120 |
| 108-31-6 | Maleic anhydride | 712.30(d), 716.120 |
| 123-33-1 | Maleic hydrazide | 799.5055(c), (d)(1), (2) |
| 109-77-3 | Malononitrile | 716.120, 799.5055(c), (d)(1), (e)(1) |
| 7785-87-7 | Manganese(II) sulfate (1:1) | 712.30(w) |
| 1313-13-9 | Manganese dioxide | 712.30(w) |
| 149-30-4 | Mercaptobenzothiazole | 712.30(m), 716.120 |
| 141-79-7 | Mesityl oxide | 712.30(i), 716.120, 799.2500 |
| 74-83-9 | Methane, bromo- | 712.30(w), 716.120 |
| 74-97-5 | Methane, bromochloro- | 716.120 |
| 75-27-4 | Methane, bromodichloro- | 716.120 |
| 74-87-3 | Methane, chloro- | 712.30(d), 716.120, 799.5055(c), (d)(2) |
| 75-45-6 | Methane, chlorodifluoro- | 712.30(w), 716.120 |
| 74-95-3 | Methane, dibromo- | 716.120, 799.5055(c), (d)(2) |
| 124-48-1 | Methane, dibromochloro- | 716.120 |
| 75-09-2 | Methane, dichloro- | 712.30(d), 716.120 |
| 75-43-4 | Methane, dichlorofluoro- | 712.30, 716.120 |
| 109-87-5 | Methane, dimethoxy- | 716.120 |
| 75-52-5 | Methane, nitro- | 712.30(w), 716.120 |
| 75-25-2 | Methane, tribromo- | 716.120, 799.5055(c), (d)(2) |
| 67-66-3 | Methane, trichloro- | 716.120 |
| 594-42-3 | Methanesulfenyl chloride, trichloride- | 799.5055(c), (d)(1), (2), (e)(1) |
| 74-93-1 | Methanethiol | 799.5055(c), (d)(1) |
| 81-21-0 | 2,4-Methano-2*H*-indeno[1,2-*b*:5,6-*b'*]bisoxirene, octahydro-. | 712.30(d) |
| 115-27-5 | 4,7-Methanoisobenzofuran-1,3-dione, 4,5,6,7,8,8-hexachloro-3a,4,7,7a-tetrahydro-. | 712.30(d) |
| 25134-21-8 | 4,7-Methanoisobenzofuran-1,3-dione,3a,4,7,7a-tetrahydromethyl-. | 712.30(d) |
| 107-98-2 | 1-Methoxy-2-propanol | 712.30, 716.120 |
| 74-87-3 | Methyl chloride | 712.30(d), 716.120, 799.5055(c), (d)(2) |
| 71-55-6 | Methyl chloroform | 712.30(d), 716.120, 799.3450 |

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|---|---|---|
| 96-37-7 | Methylcyclopentane | 712.30(o), 716.120, 799.2155 |
| 101-14-4 | 4,4'-Methylenebis(2-chloroaniline) | 704.175<br>716.120 |
| 39817-09-9 | 2,2'-Methylenebis(phenyleneoxymethyl)]bisoxirane | 712.30(d), 716.120 |
| 75-09-2 | Methylene chloride | 712.30(m), 716.120 |
| 98-82-8 | (1-Methylethyl)benzene | 712.30(d), 716.120 |
| 78-93-3 | Methyl ethyl ketone | 712.30(d), 716.120 |
| 108-10-1 | Methyl isobutyl ketone | 712.30(m), 716.120 |
| 137-20-2 | *N*-Methyl-*N*-oleoyltaurine, sodium salt | 712.30(h), 716.120 |
| 1000-82-4 | Methylolurea | 712.30(w) |
| 1313-27-5 | Molybdenum trioxide | 712.30(g), 716.120 |
| 25852-70-4 | Monobutyltin tris(isooctyl mercaptoacetate) | 712.30(g), 716.120 |
| 54849-38-6 | Monomethyltin tris(isooctyl mercaptoacetate) | 712.30(d) |
| 6033-05-2 | Morphinan-3,6-diol, 7,8-didehydro-4,5-epoxy-17-methyl-(5α,6α)-, (*Z*)-9-octadecenoate (salt). | 716.120 |
| 91-20-3 | Naphthalene | 704.43, 716.120 |
| 25586-43-0 | Naphthalene, chloro- | 704.43, 712.30(d), 716.120 |
| 90-13-1 | Naphthalene, 1-chloro- | 704.43, 716.120 |
| 91-58-7 | Naphthalene, 2-chloro- | 704.43, 716.120 |
| 70776-03-3 | Naphthalene, chloro derivatives | 704.43, 716.120 |
| 2050-69-3 | Naphthalene, 1,2-dichloro- | 704.43, 716.120 |
| 2198-75-6 | Naphthalene, 1,3-dichloro- | 704.43, 716.120 |
| 1825-31-6 | Naphthalene, 1,4-dichloro- | 704.43, 716.120 |
| 1825-30-5 | Naphthalene, 1,5-dichloro- | 704.43, 716.120 |
| 2050-72-8 | Naphthalene, 1,6-dichloro- | 704.43, 716.120 |
| 2050-73-9 | Naphthalene, 1,7-dichloro- | 704.43, 716.120 |
| 2050-74-0 | Naphthalene, 1,8-dichloro- | 704.43, 716.120 |
| 2050-75-1 | Naphthalene, 2,3-dichloro- | 704.43, 716.120 |
| 2065-70-5 | Naphthalene, 2,6-dichloro- | 704.43, 716.120 |
| 2198-77-8 | Naphthalene, 2,7-dichloro- | 716.120 |
| 3173-72-6 | Naphthalene, 1,5-diisocyanato- | 704.43, 716.120 |
| 32241-08-0 | Naphthalene, heptachloro- | 704.43, 712.30(d), 716.120 |
| 1335-87-1 | Naphthalene, hexachloro- | |
| 2234-13-1 | Naphthalene, octachloro- | 704.43, 712.30(d), 716.120 |
| 1321-64-8 | Naphthalene, pentachloro- | 704.43, 712.30(d), 716.120 |
| 1335-88-2 | Naphthalene, tetrachloro- | 704.43, 712.30(d), 716.120 |
| 1321-65-9 | Naphthalene, trichloro- | 704.43, 712.30(d), 716.120 |
| 91-92-9 | 2-Naphthalenecarboxamide, *N,N'*-(2,2'-dimethoxy[1,1'-biphenyl]-4,4'-diyl)bis[3-hydroxy-. | 716.120 |
| 2429-73-4 | 2,7-Naphthalenedisulfonic acid, 5-amino-3-[[4'-[(7-amino-1-hydroxy-3-sulfo-2-naphthalenyl) azo][1,1'-biphenyl]-4-yl]azo]-4-hydroxy-, trisodium salt. | 716.120 |
| 2429-83-6 | 2,7-Naphthalenedisulfonic acid, 4-amino-3-[[4'-[(2,4-diamino-5-methylphenyl)azo][1,1'-biphenyl]-4-yl]azo]-5-hydroxy-6-(phenylazo)-, disodium salt. | 716.120 |
| 1937-37-7 | 2,7-Naphthalenedisulfonic acid, 4-amino-3-[[4'-[2,4-diaminophenyl)azo][1,1'-biphenyl]-4-yl]azo]-5-hydroxy-6-(phenylazo)-, disodium salt. | 712.30(d), 716.120 |

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|---|---|---|
| 2586-57-4 | 1,3-Naphthalenedisulfonic acid, 4-amino-5-hydroxy-6[[4'-[(2-hydroxy-1-naphthalenyl)azo]-3,3'-dimethoxy[1,1'-biphenyl]-4-yl]azo]-, disodium salt. | 716.120 |
| 4335-09-5 | 2,7-Naphthalenedisulfonic acid, 4-amino-5-hydroxy-6-[[4'-[(4-hydroxyphenyl)azo][1,1'-biphenyl]-4-yl]azo]-3-[(4-nitrophenyl)azo]-, disodium salt | 716.120 |
| 3626-28-6 | 2,7-Naphthalenedisulfonic acid, 4-amino-5-hydroxy-3-[[4'-[(hydroxyphenyl)azo][1,1'-biphenyl]-4-yl]azo]-6-(phenylazo)-, disodium salt. | 716.120 |
| 2602-46-2 | 2,7-Naphthalenedisulfonic acid, 3,3'-[[1,1'-biphenyl]-4,4'-diylbis(azo)]bis[5-amino-4-hydroxy-, tetrasodium salt. | 712.30(d), 716.120 |
| 2610-05-1 | 1,3-Naphthalenedisulfonic acid, 6,6'-[(3,3'-dimethoxy[1,1'-biphenyl]-4,4'-diyl)bis(azo)]bis[4-amino-5-hydroxy-, tetrasodium salt. | 712.30(d), 716.120 |
| 2429-74-5 | 2,7-Naphthalenedisulfonic acid, 3,3'-[(3,3'-dimethoxy [1,1'-biphenyl]-4,4'-diyl)bis(azo)]bis[5-amino-4-hydroxy-, tetrasodium salt. | 716.120 |
| 72-57-1 | 2,7-Naphthalenedisulfonic acid, 3,3'-[(3,3'-dimethyl [1,1'-biphenyl]-4,4'-diyl)bis(azo)]bis[5-amino-4-hydroxy-, tetrasodium salt. | 712.30(d), 716.120 |
| 2150-54-1 | 2,7-Naphthalenedisulfonic acid, 3,3'-[(3,3'-dimethyl [1,1'-biphenyl]-4,4'-diyl)bis(azo)]bis [4,5-dihydroxy-, tetrasodium salt. | 716.120 |
| 3530-19-6 | 1,3-Naphthalenedisulfonic acid, 8-[[4'-[(4-ethoxy phenyl)azo][1,1'-biphenyl]-4-yl]azo]-7-hydroxy-, disodium salt. | 716.120 |
| 6358-29-8 | 1,3-Naphthalenedisulfonic acid, 8-[[4'-[(4-ethoxyphenyl)azo]-3,3'-dimethyl [1,1'-biphenyl]-4-yl]azo]-7-hydroxy-, disodium salt. | 716.120 |
| 3567-65-5 | 1,3-Naphthalenedisulfonic acid, 7-hydroxy-8-[[4'-[[4-[[(4-methylphenyl)sulfonyl]oxy]phenyl] azo][1,1'-biphenyl]-4-yl]azo]-, disodium salt. | 716.120 |
| 6449-35-0 | 1-Naphthalenesulfonic acid, 3-[[4'-[(6-amino-1-hydroxy-3-sulfo-2-naphthalenyl)azo]-3,3'-dimethoxy[1,1'-biphenyl]-4-yl]azo]-4-hydroxy-, disodium salt. | 716.120 |
| 573-58-0 | 1-Naphthalenesulfonic acid, 3,3'-[[1,1'-biphenyl]-4,4'-diylbis(azo)]bis[4-amino-, disodium salt. | 716.120 |
| 2429-71-2 | 1-Naphthalenesulfonic acid, 3,3'-[(3,3'-dimethoxy [1,1'-biphenyl]-4,4'-diyl)bis(azo)]bis[4-hydroxy-, disodium salt. | 716.120 |
| 992-59-6 | 1-Naphthalenesulfonic acid, 3,3'-[(3,3'-dimethyl [1,1'-biphenyl]-4,4'-diyl)bis(azo)bis[4-amino-, disodium salt. | 716.120 |
| 93-00-5 | 2-Napthalenesulfonic acid, 6-amino- (Bronner's acid). | 704.225(a) |
| 12001-85-3 | Naphthenic acids, zinc salts ........................................ | 716.120 |
| 26761-45-5 | Neodecanoic acid, 2,3-epoxypropyl ester Neodecanoic acid, oxiranylmethyl ester. | 712.30(d), 716.120 |
| 68958-86-1 | Nickelate (6-), [[[1,2-ethanediylbis[nitrilobis (methylene)]]tetrakis[phosphonato]](8-)]-, pentaammonium hydrogen, (*OC*-6-21)-. | 704.95 |

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|---|---|---|
| 68958-87-2 | Nickelate (6-), [[[1,2-ethanediylbis[nitrilobis(methylene)]]tetrakis[phosphonato]](8-)]-, pentapotassium hydrogen, (*OC*-6-21)-. | 704.95 |
| 68958-88-3 | Nickelate (6-), [[[1,2-ethanediylbis[nitrilobis(methylene)]]tetrakis[phosphonato]](8-)]-, pentasodium hydrogen, (*OC*-6-21)-. | 704.95 |
| 88-74-4 | 2-Nitroaniline | 712.30(d), 716.120, 799.5000 |
| 100-01-6 | 4-Nitroaniline | 712.30(d), 716.120, 799.5000 |
| 79-24-3 | Nitroethane | 712.30(w), 716.120 |
| 75-52-5 | Nitromethane | 712.30, 716.120 |
| 100-02-7 | *p*-Nitrophenol | 712.30(w), 799.5055(c), (e)(1) |
| 112-90-3 | Octadecen-1-amine, *Z*-9- | 712.30(j), 716.120 |
| 556-67-2 | Octamethylcyclotetrasiloxane | 712.30(m), 716.120, 799.5000 |
| 112-90-3 | Oleylamine | 712.30(j), 716.120 |
| 155-41-9 | 3-Oxa-9-azoniatricyclo[3.3.1.$0^{2,4}$]nonane, 7-(3-hydroxy-1-oxo-2-phenylpropoxy)-9,9-dimethyl-, bromide, [7(*S*)-(1α,2β,4β,5α,7β)]-. | 712.30(d) |
| 6106-46-3 | 3-Oxa-9-azoniatricyclo[3.3.1.$0^{2,4}$]nonane, 7-(3-hydroxy-1-oxo-2-phenylpropoxy)-9,9-dimethyl-, [7(*S*)-(1α,2β,4β,5α,7β)-, nitrate (salt). | 712.30(d) |
| 286-20-4 | 7-Oxabicyclo[4.1.0]heptane | 712.30(d) |
| 96-08-2 | 7-Oxabicyclo[4.1.0]heptane, 1-methyl-4-[2-methoxiranyl]-. | 712.30(d) |
| 470-67-7 | 7-Oxabicyclo[2.2.1]heptane, 1-methyl-4-(1-methylethyl)-. | 712.30(d) |
| 106-87-6 | 7-Oxabicyclo[4.1.0]heptane, 3-oxiranyl- | 712.30(d) |
| 145-73-3 | 7-Oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid | 712.30(d) |
| 285-67-6 | 6-Oxabicyclo[3.1.0]hexane | 712.30(d) |
| 1139-30-6 | 5-Oxatricyclo[8.2.0.$0^{4,6}$]dodecane, 4,12,12-trimethyl-9-methylene-, [1*R*-(1*R**,4*R**,6*R**,10*S**)]-. | 712.30(d) |
| 75-21-8 | Oxirane | 712.30(d), 716.120 |
| 3132-64-7 | Oxirane, bromomethyl- | 712.30(d), 716.120, 721.950 |
| 2425-79-8 | Oxirane, 2,2'-[1,4-butanediylbis(oxymethylene)]bis- | 712.30(d), 716.120 |
| 2426-08-6 | Oxirane, butoxymethyl- | 712.30(d), 716.120 |
| 106-89-8 | Oxirane, chloromethyl- | 712.30(d), 716.120 |
| 2855-19-8 | Oxirane, decyl- | 712.30(d), 716.120 |
| 35243-89-1 | Oxirane, [(1,2-dibromopropoxy)methyl]- | 716.120 |
| 20217-01-0 | Oxirane, [(2,4-dibromophenoxy)methyl]- | 716.120 |
| 558-30-5 | Oxirane, 2,2-dimethyl- | 716.120 |
| 3266-23-7 | Oxirane, 2,3-dimethyl- | 716.120 |
| 7665-72-7 | Oxirane, [(1,1-dimethylethoxy)methyl]- | 716.120 |
| 3101-60-8 | Oxirane, [[4-(1,1-dimethylethyl)phenoxy]methyl]- | 712.30(d), 716.120 |
| 17557-23-2 | Oxirane, 2,2'-[(2,2-dimethyl-1,3-propanediyl)bis(oxymethylene)]bis-. | 716.120 |
| 3234-28-4 | Oxirane, dodecyl- | 716.120 |
| 2461-18-9 | Oxirane, [(dodecyloxy)methyl]- | 712.30(d), 716.120 |
| 2224-15-9 | Oxirane, 2,2'-[1,2-ethanediylbis(oxymethylene)]bis- | 716.120 |
| 7328-97-4 | Oxirane,2,2',2'',2'''-[1,2-ethanediylidenetetrakis (4,1-phenyleneoxymeth ylene)]tetrakis-. | 716.120 |
| 930-22-3 | Oxirane, ethenyl- | 716.120 |
| 4016-11-9 | Oxirane, ethoxymethyl- | 712.30(d), 716.120 |

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| 106-88-7 | Oxirane, ethyl- | 712.30(d), 716.120 |
| 2461-15-6 | Oxirane, [[(2-ethylhexyl)oxy]methyl]- | 716.120 |
| 67860-04-2 | Oxirane, heptadecyl- | 716.120 |
| 7390-81-0 | Oxirane, hexadecyl- | 716.120 |
| 15965-99-8 | Oxirane, [(hexadecyloxy)methyl]- | 716.120 |
| 68959-23-9 | Oxirane, 2,2',2''-[1,2,6-hexanetriyltris (oxymethylene)]tris-. | 716.120 |
| 930-37-0 | Oxirane, methoxymethyl- | 712.30(d), 716.120 |
| 75-56-9 | Oxirane, methyl- | 712.30(d), 716.120, 799.3450 |
| 54208-63-8 | Oxirane, 2,2'-[methylenebis(2,1-phenyleneoxymethylene)]bis-. | 716.120 |
| 4016-14-2 | Oxirane, [(1-methylethoxy)methyl]- | 712.30(d), 716.120 |
| 71033-08-4 | Oxirane, 2,2'-[(methylethylidene)bis[4,1-phenyleneoxy[1-(butoxymethyl)-2,1-ethanediyl]oxymethylene]]bis-. | 716.120 |
| 1675-54-3 | Oxirane, 2,2'-[(1-methylethylidene)bis(4,1-phenyleneoxy methylene)]bis-. | 716.120 |
| 25085-99-8 | Oxirane, 2,2'-[(1-methylethylidene)bis(4,1-phenyleneoxy methylene)]bis-, homopolymer. | 712.30(d), 716.120 |
| 72319-24-5 | Oxirane, 2,2'-[(1-methylethylidene)bis[4,1-phenyleneoxy-3,1-propanediyloxy-4,1-phenyleneoxymethylene]]bis-. | 716.120 |
| 26447-14-3 | Oxirane, [(methylphenoxy)methyl]- | 712.30(d), 716.120 |
| 2210-79-9 | Oxirane, [(2-methylphenoxy)methyl]- | 716.120 |
| 61578-04-9 | Oxirane, [[4-(1-methyl-1-phenylethyl)phenoxy]methyl]-. | 716.120 |
| 68081-84-5 | Oxirane, mono[(C$_{10-16}$-alkyloxy)methyl] derivatives | 716.120 |
| 68609-97-2 | Oxirane, mono[(C$_{12-14}$-alkyloxy)methyl] derivatives | 716.120 |
| 68609-96-1 | Oxirane, mono[(C$_{8-10}$-alkyloxy)methyl] derivatives | 716.120 |
| 68987-80-4 | Oxirane, mono[C$_{8-12}$-alkyloxy)methyl] derivatives | 716.120 |
| 5255-75-4 | Oxirane, [(4-nitrophenoxy)methyl]- | 716.120 |
| 60501-41-9 | Oxirane, [(9-octadecenyloxy)methyl]-, (*Z*)- | 712.30(e), 716.120 |
| 16245-97-9 | Oxirane, [(octadecyloxy)methyl]- | 716.120 |
| 2404-44-6 | Oxirane, octyl- | 716.120 |
| 13561-08-5 | Oxirane, 2,2'-(oxiranylmethoxy)-1,3-phenylene]bis (methylene)]bis-. | 712.30(d), 716.120 |
| 67786-03-2 | Oxirane, 2,2'-[[[2-oxiranylmethoxy)phenyl]methylene] bis(4,1-phenyleneoxymethylene)]bis-. | 716.120 |
| 2238-07-5 | Oxirane, 2,2'-[oxybis(methylene)]bis- | 712.30(d), 716.120 |
| 22092-38-2 | Oxirane, pentadecyl- | 716.120 |
| 122-60-1 | Oxirane, (phenoxymethyl)- | 712.30(d), 716.120 |
| 96-09-3 | Oxirane, phenyl- | 712.30(d) |
| 101-90-6 | Oxirane, 2,2'-[1,3-phenylenebis(oxymethylene)]bis- | 712.30(d), 716.120 |
| 2425-01-6 | Oxirane, 2,2'-[1,4-phenylenebis(oxymethylene)]bis- | 716.120 |
| 13236-02-7 | Oxirane, 2,2',2''-[1,2,3-propanetriyltris (oxymethylene)]tris-. | 712.30(d), 716.120 |
| 106-92-3 | Oxirane, [(2-propenyloxy)methyl]- | 712.30(d), 716.120 |
| 68517-02-2 | Oxirane, 2,2',2''-[propylidynetris(4,1-phenyleneoxy methylene)]tris-. | 716.120 |
| 7320-37-8 | Oxirane, tetradecyl- | 712.30(d), 716.120 |
| 38954-75-5 | Oxirane, [(tetradecyloxy)methyl]- | 716.120 |
| 3083-25-8 | Oxirane, (2,2,2-trichloroethyl)- | 712.30(d), 716.120, 721.975 |

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|---|---|---|
| 38565-52-5 | Oxirane, (2,2,3,3,4,4,5,5,6,6,7,7,7-trideca fluoroheptyl)-. | 716.120 |
| 18633-25-5 | Oxirane, tridecyl- | 716.120 |
| 428-59-1 | Oxirane, trifluoro(trifluoromethyl)- | 712.30(d), 716.120, 721.1175 |
| 77-83-8 | Oxiranecarboxylic acid, 3-methyl-3-phenyl-, ethyl ester. | 712.30(d), 716.120 |
| 121-39-1 | Oxiranecarboxylic acid, 3-phenyl-, ethyl ester | 712.30(d), 716.120 |
| 5026-74-4 | Oxiranemethanamine, *N*-[4-(oxiranylmethoxy)phenyl]-*N*-(oxiranylmethyl)-. | 716.120 |
| 556-52-5 | Oxiranemethanol | 712.30(d), 716.120 |
| 106-83-2 | Oxiraneoctanoic acid, 3-octyl-, butyl ester | 712.30(d) |
| 141-38-8 | Oxiraneoctanoic acid, 3-octyl-, 2-ethylhexyl ester | 712.30(d) |
| 106-84-3 | Oxiraneoctanoic acid, 3-octyl-, octyl ester | 712.30(d) |
| 7647-10-1 | Palladium(II) chloride | 712.30(w) |
| 63449-39-8 | Paraffin waxes and hydrocarbon waxes, chlorinated | 716.120 |
| 608-93-5 | Pentachlorobenzene | 712.30(d), 716.120, 799.5055(c) |
| 76-01-7 | Pentachloroethane | 721.497, 799.5055(c) |
| 540-84-1 | Pentane, 2,2,4-trimethyl- | 716.120 |
| 108-10-1 | 2-Pentanone, 4-methyl- | 712.80(d), 716.120 |
| 85-01-8 | Phenanthrene | 704.225(a) |
| 108-95-2 | Phenol | 716.120 |
| 88-04-0 | Phenol, 4-chloro-3,5-dimethyl- | 712.30(w), 716.120 |
| 120-32-1 | Phenol, 4-chloro-2-(phenylmethyl)- | 712.30, 716.120 |
| 137-09-7 | Phenol, 2,4-diamino-, dihydrochloride | 712.30(f), 716.120 |
| 15872-73-8 | Phenol, 2,4-diamino-6-methyl- | 712.30(f), 716.120 |
| 65879-44-9 | Phenol, 4,6-diamino-2-methyl-, hydrochloride | 712.30(f), 716.120 |
| 472-41-3 | Phenol, 4-(3,4-dihydro-2,2,4-trimethyl-2*H*-1-benzopyran-4-yl)-. | 716.120 |
| 1300-71-6 | Phenol, dimethyl- | 716.120 |
| 25155-23-1 | Phenol, dimethyl-, phosphate (3:1) | 712.30(d), 716.120 |
| 78-33-1 | Phenol, (4-(1,1-dimethylethyl)-, phosphate (3:1) | 712.30(d), 716.120 |
| 1319-77-3 | Phenol, methyl- | 712.30(d) |
| 95-48-7 | Phenol, 2-methyl- | 712.30(d), 716.120, 799.1250 |
| 108-39-4 | Phenol, 3-methyl- | 712.30(d), 716.120, 799.1250 |
| 106-44-5 | Phenol, 4-methyl- | 712.30(d), 716.120, 799.1250 |
| 97-23-4 | Phenol, 2,2'-methylenebis[4-chloro- | 716.120 |
| 25168-06-3 | Phenol, (1-methylethyl)- | 712.30, 716.120 |
| 80-05-7 | Phenol, 4,4'-(1-methylethylidene)bis- | 712.30(k), 716.120, 799.940 |
| 79-94-7 | Phenol, 4,4'-(1-methylethylidene)bis[2,6-dibromo- | 799.4000 |
| 100-02-7 | Phenol, 4-nitro- | 712.30, 716.120, 799.5055(c) |
| 140-66-9 | Phenol, 4-(1,1,3,3-tetramethylbutyl)- | 712.30(g), 716.120 |
| 97-18-7 | Phenol, 2,2'-thiobis[4,6-dichloro- | 716.120 |
| 118-79-6 | Phenol, 2,4,6-tribromo- | 712.30(d) |
| 92-84-2 | 10*H*-Phenothiazine | 716.120 |
| 122-99-6 | 2-Phenoxyethanol | 712.30(h), 716.120 |
| 106-50-3 | *p*-Phenylenediamine | 712.30(d), 716.120 |
| 603-35-0 | Phosphine, triphenyl- | 712.30(d) |

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|---|---|---|
| 1429-50-1 | Phosphonic acid, [1,2-ethanediylbis[nitrilobis(methylene)]]tetrakis- (EDTMPA). | 704.95 |
| 57011-27-5 | Phosphonic acid, [1,2-ethanediylbis[nitrilobis(methylene)]]tetrakis-, ammonium salt. | 704.95 |
| 15142-96-8 | Phosphonic acid, [1,2-ethanediylbis[nitrilobis(methylene)]]tetrakis-, hexasodium salt. | 704.95 |
| 68901-17-7 | Phosphonic acid, [1,2-ethanediylbis[nitrilobis(methylene)]]tetrakis-, octaammonium salt. | 704.95 |
| 34274-30-1 | Phosphonic acid, [1,2-ethanediylbis[nitrilobis(methylene)]]tetrakis-, potassium salt. | 704.95 |
| 68188-96-5 | Phosphonic acid, [1,2-ethanediylbis[nitrilobis(methylene)]]tetrakis-, tetrapotassium salt. | 704.95 |
| 66108-37-0 | Phosphoric acid, 2,2-bis(bromomethyl)-3-chloropropylbis[2-chloro-1-(chloromethyl)ethyl] ester. | 712.30(d) |
| 2528-36-1 | Phosphoric acid, dibutyl phenyl ester | 712.30(d), 716.120 |
| 51363-64-5 | Phosphoric acid, diisodecyl phenyl ester | 712.30(d), 716.120 |
| 56803-37-3 | Phosphoric acid, (1,1-dimethylethyl)phenyl diphenyl ester. | 712.30(d), 716.120 |
| 1241-94-7 | Phosphoric acid, 2-ethylhexyl diphenyl ester | 712.30(d), 716.120 |
| 29761-21-5 | Phosphoric acid, isodecyl diphenyl ester | 712.30(d), 716.120 |
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| 26444-49-5 | Phosphoric acid, methylphenyl diphenyl ester | 712.30(d), 716.120 |
| 34364-42-6 | Phosphoric acid, (1-methyl-1-phenylethyl)phenyl diphenyl ester. | 716.120 |
| 126-73-8 | Phosphoric acid, tributyl ester | 712.30(q), 716.120 |
| 115-86-6 | Phosphoric acid, triphenyl ester | 712.30(d), 716.120 |
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| 563-04-2 | Phosphoric acid, tris(3-methylphenyl) ester | 716.120 |
| 78-32-0 | Phosphoric acid, tris(4-methylphenyl) ester | 712.30(d), 716.120 |
| 3288-58-2 | Phosphorodithioic acid, *O,O*-diethyl-*S*-methyl ester | 716.120 |
| 7723-14-0 | Phosphorous | 712.30(w) |
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| 121-45-9 | Phosphorous acid, trimethyl ester | 712.30(d) |
| 11126-42-4 | Polychlorinated *o*-terphenyl | 704.85, 716.120 |
| 61788-33-8 | Polychlorinated *p*-terphenyl | 704.85, 716.120 |
| 68389-89-9 | Poly(oxy-1,2-ethanediyl), α-[2-[bis(2-aminoethyl)methyl ammonio]ethyl]-ω-hydroxy-, *N,N'*-bis(hydrogenated tallow acyl) derivatives, methyl sulfates (salts). | 712.30(w), 716.120(a)(1) |
| 70914-09-9 | Poly(oxy-1,2-ethanediyl), α-[2-[bis(2-aminoethyl)methyl ammonio]ethyl]-ω -hydroxy-, *N,N'*-di-C14-18 acyl derivatives, methyl sulfates (salts). | 712.30(w), 716.120(a)(1) |
| 68389-88-8 | Poly(oxy-1,2-ethanediyl), α-[2-[bis(2-aminoethyl)methyl ammonio]ethyl]-ω-hydroxy-, *N,N'*-dicoco acyl derivatives, methyl sulfates (salts). | 712.30(w), 716.120(a)(1) |

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| 68410-69-5 | Poly(oxy-1,2-ethanediyl), α-[2-[bis(2-aminoethyl)methyl ammonio]ethyl]-ω-hydroxy-, *N,N'*-ditallow acyl derivatives, methyl sulfates (salts). | 712.30(w), 716.120(a)(1) |
| 68554-06-3 | Poly(oxy-1,2-ethanediyl), α-[3-[bis(2-aminoethyl)methyl ammonio]-2-hydroxypropyl]-ω-hydroxy-, *N*-coco acyl derivatives, methyl sulfates (salts). | 712.30(w), 716.120(a)(1) |
| 69943-75-5 | Poly(oxy-1,2-ethanediyl), α-[4-(oxiranylmethoxy)benzoyl]-ω-[[4-(oxiranylmethoxy)benzoyl]oxy]-. | 716.120 |
| 68413-04-7 | Poly[oxy(methyl-1,2-ethanediyl)], α-[2-[bis(2-amino ethyl)methylammonio]methylethyl]-ω-hydroxy-, *N,N'*- ditallow acyl derivatives, methyl sulfates (salts). | 712.30(w), 716.120(a)(1) |
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| 96-12-8 | Propane, 1,2-dibromo-3-chloro- | 712.30(d) |
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| 18495-30-2 | Propane, 1,1,2,3-tetrachloro- | 716.120 |
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| 124-16-3 | 2-Propanol, 1-(2-butoxyethoxy)- | 712.30, 716.120 |
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| 25498-49-1 | Propanol, [2-(2-methoxymethylethoxy)methylethoxy]-. | 712.30, 716.120 |
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| 563-58-6 | 1-Propene, 1,1-dichloro- | 716.120 |
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| 116-15-4 | 1-Propene, 1,1,2,3,3,3-hexafluoro- | 712.30(d), 716.120, 799.1700(a)(1) |
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| 106-63-8 | 2-Propenoic acid, 2-methylpropyl ester | 712.30(d) |
| 25584-83-2 | 2-Propenoic acid, monoester with 1,2-propanediol | 712.30(d) |
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| 16715-83-6 | 2-Propenoic acid, 2-methyl-, 2-[bis(1-methylethyl) amino] ethyl ester. | 712.30(d) |
| 2082-81-7 | 2-Propenoic acid, 2-methyl-, 1,4-butanediyl ester | 712.30(d) |
| 97-88-1 | 2-Propenoic acid, 2-methyl-, butyl ester | 712.30(d), 716.120 |
| 101-43-9 | 2-Propenoic acid, 2-methyl-, cyclohexyl ester | 712.30(d) |
| 105-16-8 | 2-Propenoic acid, 2-methyl-, 2-(diethylamino) ethyl ester. | 712.30(d) |
| 2867-47-2 | 2-Propenoic acid, 2-methyl-, 2-(dimethylamino)ethyl ester. | 712.30(d) |
| 3775-90-4 | 2-Propenoic acid, 2-methyl-, 2-[(1,1,-dimethylethyl) amino]ethyl ester. | 712.30(d) |
| 142-90-5 | 2-Propenoic acid, 2-methyl-, dodecyl ester | 712.30(d) |
| 97-90-5 | 2-Propenoic acid, 2-methyl-, 1,2-ethanediyl ester | 712.30(d) |
| 109-16-0 | 2-Propenoic acid, 2-methyl-, 1,2-ethanediylbis(oxy-2,1- ethanediyl) ester. | 712.30(d) |
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| 2530-85-0 | 2-Propenoic acid, 2-methyl-, 3-(trimethoxysilyl)propyl ester. | 712.30(d) |
| 7534-94-3 | 2-Propenoic acid, 2-methyl-, 1,7,7-trimethylbicyclo [2.2.1]hept-2-yl ester, *exo*-. | 712.30(d) |
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| 5344-82-1 | Thiourea, (2-chlorophenyl)- | 716.120 |
| 108-88-3 | Toluene | 712.30(d), 716.120 |
| 29385-43-1 | Tolyl triazole | 712.30, 716.120 |
| 3083-25-8 | Trichlorobutylene oxide | 712.30(d), 716.120, 721.975 |
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